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Overexpression of HER-2/*neu* (a member of the EGF receptor family) and inactivation of estrogen receptor (ER) or the tumor suppressor gene, Rb, are known to be involved in the development of human breast cancer. Expression of HER-2/*neu* can be regulated by ER or Rb. In addition, expression of other EGF receptor family genes (EGF receptor, HER-3 and HER-4) and Heregulin, a ligand for the HER-3 and HER-4 encoded receptor has also been found in some breast cancer cells and may contribute to malignant transformation of breast cancer.The current progress report focuses on the role of the EGF receptor family genes, Heregulin, ER, Rb, and their interrelationship in breast cancer. The technical objectives are: **(1) Systematic studies on the expression of EGF receptor family genes, Heregulin, and ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival. (2) Potential paracrine and autocrine loops for EGF receptor family molecules and Heregulin. (3) Effects of ER on malignant transformation phenotypes of breast cancer cells. (4) Effects of Rb on malignant transformation phenotypes of HER-2/*neu*-overexpressing breast cancer cells.** Expression of EGF receptor family genes, Heregulin, and ER in the same breast tumor specimens were examined by immunohistochemical staining, and western blotting. The relationship between expression of these molecules, tumor grades, and patient's survival will be evaluated Using gene transfer technique, Heregulin, ER or Rb gene are being introduced into HER-2/*neu*-expressing breast cancer cells. The effect on their malignant transformations will be examined. *This project may help to develop a more reliable molecular prognostic strategy and to understand how interactions among multiple genetic factors are involved in the development of breast cancer.*

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INTRODUCTION

A. Background

The data from the Surveillance, Epidemiology, and End Results (SEER) Program indicates breast cancer remains a major cause of death in this country (1). It was estimated that approximately 180,000 new cases of breast cancer will be diagnosed in the United States in 1995, and 46,000 women will die from this disease. Many oncogenes and tumor suppressor genes were shown to be involved in the development of breast cancer. The clinical value of each of these genes in prognostic and potential therapeutic applications has been studied to some extent (2). Yet, none of these molecular markers alone was found to be a better prognosis factor than the prognosis factors currently used for breast cancer patients (e.g. number of metastatic lymph nodes). **Identification of a set of related genes that are involved in breast cancer may be critical to develop a better molecular prognostic strategy. Studies on the role of a set of related genes and their interrelationship in breast cancer may also provide a more productive avenue to understand the basic biology of breast cancer cells.** Loss of estrogen receptor (ER) expression or function has been known for a long time to be associated with poor prognosis for breast cancer patients. The *HER-2/neu* oncogene encodes a growth factor receptor-like molecule and overexpression of *HER-2/neu* is also reported to correlate positively with poor survival for breast cancer patients. Some studies, including our own, demonstrate that estrogen-stimulated ER can repress *HER-2/neu* overexpression, suggesting a possible causal relationship between *HER-2/neu* overexpression and non-function of ER in breast cancer cells. We also have recently found that the tumor suppressor gene, Rb, can repress *HER-2/neu* expression. These studies raised an interesting possibility that ER and Rb may suppress the effects on the malignant transformation phenotype induced by *HER-2/neu* overexpression. The Rb gene is known to be inactivated in approximately 20% of breast tumors. It is not yet clear whether Rb might have prognosis value in breast cancer.

A-1. The *HER-2/neu* Proto-oncogene Encoding an Epidermal Growth Factor (EGF) Receptor-related Protein is a Potent Transforming Oncogene

The rat *neu* oncogene was a transforming gene originally isolated from rat neuroblastoma and later its cellular counterpart, the normal *neu* gene, was also isolated from rat and human libraries (3-6). Detailed structural and functional analysis of the transforming rat *neu* and the normal *neu* genes (*neu* proto-oncogene) indicates that a single point mutation in the transmembrane region is responsible for the conversion of the normal *neu* gene into a transforming *neu* oncogene (7). The human homologue (HER-2 or c-*erb*B-2) of rat *neu* oncogene was isolated based on its homology to chicken v-*erb*B gene (HER-2 represents Human EGF Receptor-2 and c-*erb*B-2 means the second gene homologous to v-*erb*B; EGF receptor gene was the first). It is now known that *neu*, HER-2, and c-*erb*B2 are the same gene. We will use *HER-2/neu* to represent this gene since *HER-2/neu* seems to be the most frequently used in the literature. Structural comparison between the *HER-2/neu*-encoded p185 protein and EGF receptor revealed significant sequence homology and identical gross structural organization including ligand-binding, transmembrane, and tyrosine-kinase domains between these two proteins (5-8). Both EGF receptor and p185 proteins can form either homodimers or heterodimers (9-11). The homodimer is believed to be an active receptor form for ligand binding. Two other EGF receptor-related genes, HER-3 and HER-4, have also recently been cloned. Recent experimental evidence indicates that these four EGF receptor-related proteins are able to form heterodimers among each other, suggesting different combination of heterodimers may interact with distinct ligands and induce signal transduction in specific cell type (12,13). A molecule (named Heregulin or NDF), thought to be a ligand for HER-2/*neu*, recently has been shown to be a ligand for HER-3 and HER-4 (14-19). Heregulin is able to bind HER-3 and HER-4 homodimers but cannot bind to HER-2/*neu* or EGF receptor homodimers. However, Heregulin is able to interact with HER-2/*neu* through heterodimerization of HER-2/*neu* with HER-3 or with HER-4 (12,13). Since Heregulin and the four EGF receptor-related proteins (EGF receptor, HER-2/*neu*, HER-3 and HER-4) can interact with each other, it is likely that they may all be involved in the development of breast cancer. In this Progress Report, we have begun to investigate their expression in breast tumor specimen by immunohistochemical staining.

A-2. Amplification/Overexpression of the Human *HER-2/neu* Gene is Frequently Found in Human Cancers including Breast Cancer.

Unlike the rat *neu* oncogene that is activated by a single point mutation, the human *HER-2/*neu gene is activated by overexpression in human cancers. Amplification/overexpression of the *HER-2/neu* gene was first found in approximately 30% of human breast cancers (20-25) and later in many other human cancers (31-36). In the case of breast, ovarian, lung and gastric cancers, several reports further indicated that *HER-2/neu* overexpression correlates with a poor survival rate (25-31), suggesting that *HER-2/neu* overexpression may be used as a prognosis factor (25-27, 20, 22). However, some studies disagree that *HER-2/neu* overexpression can be a poor prognostic factor in breast cancer (32-34). Although the discrepancy could be caused by reasons such as patient population and treatment differences, the methods and reagents used for detection of *HER-2/neu* overexpression and the way tumor specimens were collected, other more significant reasons may also contribute to this discrepancy. Considering the fact that the development of breast cancer requires multi-step activations, it is possible that *HER-2/neu* overexpression alone may not be an ideal prognosis factor for breast cancer patients. Combined data from the expression of multiple oncogenes and tumor suppressor genes involved in breast cancer may provide a more accurate prognosis. Both ER and Rb are shown to be involved in breast cancer and our studies also indicate that they can regulate *HER-2/neu* expression (see below). In addition, the other EGF receptor-related molecules and Heregulin that can interact with HER-2/*neu* as discussed earlier, may also be involved in the development of breast cancer.

A-3. Non-function of ER May Contribute to *HER-2/neu* Overexpression in Some Breast Cancer

Steroid hormones play an essential part in regulating the growth of both normal and neoplastic breast cells. Specifically, estrogen has a marked effect on the proliferation of breast cells *in vivo* and *in vitro*. Although the mechanisms by which estradiol (E2) induces proliferation in estrogen receptor (ER) positive breast cells are incompletely defined, modulation in expression of certain growth related cellular proto-oncogenes by estradiol stimulated estrogen receptor (E2/ER) has been well-described using cell lines established from human breast tumors. The function of ER in breast cancer is unclear; however, the significant correlation between loss of functional estrogen receptor and poor patient prognosis is very well described (35,36). Evidence that ER may play a role in the regulation of *HER-2/neu* expression comes from several diverse observations. Several studies of human breast tumor tissue specimens have shown an inverse relationship between ER and *HER-2/neu* expression (37-40). Furthermore, during development of rat mammary glands, expression of *HER-2/neu* is inversely related to ER status (41). Those breast cancer cell lines with the highest levels of *HER-2/neu* overexpression are generally ER negative or have very low levels of estrogen receptor. We and others have previously shown that ER through estrogen stimulation can negatively regulate the expression of *HER-2/neu* in ER positive but not ER negative breast cancer cell lines (42-44). We further demonstrated that the ER-mediated *HER-2/neu* repression can occur at the transcriptional level (44). The result suggests that *HER-2/neu* overexpression may be caused by inactivation of ER in some breast tumors. It also raises an interesting possibility that expression of ER in the ER negative and *HER-2/neu*-overexpressing breast cancer cells may suppress malignant transformation induced by *HER-2/neu* overexpression. If this indeed is correct, it may provide an interpretation for a well-known clinical phenomenon, namely, some ER positive breast cancer patients do not respond to hormone treatment such as Tamoxifen (TAM), an estrogen antagonist. **Our hypothesis is that ER positive breast tumors with *HER-2/neu* overexpression may not respond to TAM treatment since TAM competes with estrogen to interact with ER, and in one way blocks the stimulating effect of tumor cell growth (Estrogen can stimulate growth of ER positive cells), but in another way enhance *HER-2/neu* overexpression, which enables the tumor cells to become more malignant. In this Progress Report, we have completed the study indicating that ER can suppress cellular transformation of HER-2/*neu*-overexpressing breast cancer cells through E2/ER-mediated HER-2/*neu* repression.**

A-4. The Tumor Suppressor Gene, Rb, May Suppress Tumorigenicity of Human Breast Cancer Cells with *HER-2/neu* Overexpression.

The retinoblastoma susceptibility gene (Rb) is a well-characterized tumor suppressor gene (45). The existence of this gene was initially predicted based upon genetic predispositions to certain pediatric malignancies (46). Tumor formation or transformation occurs when these genes are inactivated, suggesting that their normal function is to limit cellular proliferation. Inactivation or deletion of Rb has been found in a variety of human cancers including breast cancer (45). Using retroviral-mediated gene transfer, it has been shown that the Rb gene can suppress tumor formation of retinoblastoma, osteosarcoma, and breast carcinoma in which the endogenous Rb gene is inactivated (47-49).

The Rb gene encodes a 105kDa protein (RB) and is known to form a protein complex with adenovirus E1A protein as well as large T (LT) antigen of SV40 virus and E7 protein of papilloma virus (50-52). It is believed that the DNA virus-associated proteins such as E1A, LT and E7 may inactivate the RB function through RB-E1A (or LT, E7) complex. Biochemically, RB can function as a transcriptional factor that can regulate transcription of cellular genes including c-*myc*, TGF-b, c-*fos* and *HER-2/neu* (53-56). More recently, RB has been shown to form a protein complex with a DNA-binding protein E2F and may, therefore, act as a transcriptional factor by complexing with other factors (57,58). We have previously demonstrated that E1A gene products inhibit *HER-2/neu* expression in both rodent and human breast cancer cells (59-61). The results allow us to further discover that the E1A gene functions as a tumor suppressor gene for the *HER-2/neu*-overexpressing tumor cells through transcriptional repression of *HER-2/neu* gene. Since E1A and RB proteins can form E1A-RB protein complexes and it is believed that E1A proteins may inactivate RB function through E1A-RB complexes, our results which clearly demonstrated that E1A gene is a tumor suppressor gene for the *HER-2/neu* transformed cells seemed to be surprising at the beginning. However, it has recently been shown that E1A can stabilize the tumor suppressor, p53, and induce cell program death (Apoptosis) (62-65). This property strongly supports tumor suppressor function of E1A. We have now shown that RB alone can also inhibit *HER-2/neu* transformation through transcriptional repression (56). The effect of RB-E1A complexes on *HER-2/neu* expression is very complex as RB can enhance E1A-mediated *HER-2/neu* repression in some cell types but not in other cell types (our unpublished observation). Our preliminary results suggest an interesting phenomenon: that E1A and RB may cooperate to enhance a biological activity in one cell type while antagonizing each other to suppress a biological function in another cell type. This complex issue will not be addressed in the current Progress Report. Instead, we will focus on the RB effect on *HER-2/neu*-overexpressing breast cancer cells. If RB can also suppress malignant transformation of *HER-2/neu*-overexpressing cancer cells, the tumor suppressor function of the Rb gene will not be limited only to the Rb-defective tumors and may extend to the tumors with *HER-2/neu* overexpression. This will become an important issue when the gene therapy technique is developed well enough to allow delivery of the Rb gene into patient's cancer cells.

B. THE PURPOSES

The major purposes are:

B-1. Systematic studies on the expression of EGF receptor family, Heregulin, ER in breast tumor specimens and correlation of the expression with tumor stages and patient survival.

Our hypothesis is that the combination of EGF receptor family, Heregulin, ER may be a better prognosis factor than each of these molecules individually. Therefore, expression of EGF receptor family, Heregulin, ER, in the same breast tumor specimens will be examined by immuno-histochemical staining, and western blots. The relationship between expression of these molecules, tumor grades and patients' survival will be evaluated.

B-2. Potential paracrine and autocrine interactions between EGF receptor family and Heregulin in breast cancer cells.
Potential paracrine and autocrine loops between EGF receptor family and Heregulin ligand will be tested by using expression vectors and model cell lines. Effects of Heregulin on transformation phenotypes of breast cancer cells will be examined by growth properties, soft agar colonization assay, subcutaneous (s.c.) tumorigenicity and intraperitoneal (i.p.) survival assays.

B-3. Effects of ER on malignant transformation phenotypes of *HER-2/neu*-overexpressing breast cancer cells.
Since we have found that estrogen-stimulated ER can repress *HER-2/neu* gene expression, ER expression vectors will be used to modulate *HER-2/neu* expression in *HER-2/neu* overexpressing breast cancer cells. The effect of ER on transformation phenotypes of *HER-2/neu* overexpressing breast cancer cells will be examined. The potential effects of Tamoxifen, an estrogen antagonist, will also be tested in this system.

B-4. Effects of Rb on malignant transformation phenotypes of breast cancer cells.
The Rb-expression vectors will be introduced into the breast cancer cells. The effects of Rb on *HER-2/neu* expression and transformation phenotypes will be analyzed. Potential relationship among Rb, ER and *HER-2/neu* will also be examined.

BODY

1. Systematic studies on the expression of EGF receptor family, Heregulin, ER and Rb.

Heregulin was originally thought to be a ligand for HER-2/*neu* (12-14). However, more recent data indicate that Heregulin is a ligand for HER-3 and HER-4 (also known as c-*erb*B3 and c-*erb*B4) that are two recently identified EGF receptor family genes. Since HER-3 and HER-4 can form heterodimers with HER-2/*neu* and interactions between Heregulin and HER-2/*neu* is most likely through HER-3:HER-2/*neu* dimer or HER-4:HER-2/*neu* dimer (12-13), it is possible that HER-3 and HER-4 may also contribute to breast cancer. In addition, overexpression of EGF receptor is known to be involved in human breast cancer. In this Progress Report, we will expand our expression study in breast tumor tissues for all 4 EGF receptor family (EGF receptor, HER-2/neu, HER-3 and HER-4).

We have used specific antibodies for EGF receptor, HER-2/*neu*, HER-3, HER-4, and Heregulin to stain the archival paraffin-embedded sections. Up to now, 200 tumor sections have been completed for EGF receptor HER-2/*neu* and HER-3. One set of representative data was shown in Fig. 1 (EGF receptor), Fig. 2 (HER-2/*neu*) and Fig. 3 (HER-3). In these cases, we can identify tumors with high, intermediate, low and negative expression. Quantitation of antibody staining is justified by relative intensity of positive staining by professional pathologist. The relationship between the expression level with ER status tumor grades, patient survival and other clinical status is currently under investigation. One example in which 123 cases with HER-2/*neu* expression status were analyzed was shown in Table 1. We are continuing to collect these clinical status from our archival medical record. Table 1 is an example to compare the clinical status with HER-2/*neu* expression. Since the same tumor specimen were screened for multiple gene expression such as EGF receptor, HER-3, etc. Once the data is completed, the analysis can be easily compared with each individual gene expression and multiple variant analysis can also be analyzed. We will continue to determine EGF receptor family and Heregulin expression levels in these tumor sections.

Involvement of Rb in human cancer is primarily regulated by the non-function of Rb protein that could be derived from deletion or mutation of the Rb gene. In some cases, the aberrant Rb protein can still be expressed in tumors and therefore scored as "positive" by immunohistochemical staining. Although western blotting analysis may detect aberrant form of Rb protein, it may not be able to distinguish difference between single-point-mutated Rb and wild-type Rb protein. Therefore, immunohistochemical staining for Rb protein will not be a very informative strategy to detect inactivation of Rb in tumor tissues (This was also discussed in the original grant proposal.). As mentioned earlier, the newly identified molecules, HER-3 and HER-4, like HER-2/*neu*, belong to the EGF receptor family and interactions between Heregulin and HER-2/*neu* require existence of HER-3 or HER-4 (most likely through heterodimer between HER-3 and HER-2/*neu* or HER-4 and HER-2/*neu*). Overexpression of EGF receptor can be easily identified by immunohistochemical staining (e.g. Please see Fig. 1-3). We feel it will be more informative to analyze EGF receptor family expression than to analyze Rb expression, since Rb expression detected by immunohistochemical staining does not really reflect its contribution to tumor development (as discussed earlier). We would, therefore, like to focus on EGF receptor family and Heregulin in the expression studies. The ER status will be obtained from medical record (e.g. Please Table 1).

2. Potential Paracrine and Autocrine Interactions between HER-2/*neu* and Heregulin in Breast Cancer Cells.

We have previously completed the construction of Heregulin-expression vectors and anti-sense Heregulin plasmids and also identified several breast cancer cell lines that express HER-3 and also overexpress HER-2/*neu*. These include MDA-MB-453, MDA-MB-361, SKBr3. MDA-MB-231 and MDA-MB-435 are known to express only basal level of HER-2/*neu*. To establish stable transfectants, Heregulin-expression vector has been transfected into HER-3 positive and HER-2/*neu*-overexpressing cells and anti-sense construct into Heregulin-expressing cells (MDA-MB-231). Ten G418-resistant colonies have been selected for Heregulin transfectants (MDA-MB-453 as recipient cells). Expression of Heregulin is currently underway by using western blot analysis. Multiple G418-resistant colonies were also observed after anti-sense Heregulin construct was transfected into MDA-MB-231 cells. These colonies are currently under selection and will be expanded for analysis of down-regulation of Heregulin expression.

3. Effects of ER on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells.

Using our ER-transfectants (59), we have previously shown that estrogen indeed suppresses transformation phenotype of ER+, HER-2/*neu* breast cancer cells as predicted in our original proposal. A manuscript described this study has been published in Oncology Report. A reprint is attached in lieu of detailed description.

The abstract of this paper is described as follows:

The mechanisms by which breast cancers progress to hormone independence does not always require the loss of estrogen receptor (ER) expression or function. Cellular alterations that disturb the normal pathway of estrogen-regulated growth may contribute to a state of hormone independence. We and others have described an inverse relationship between estrogen stimulation of ER+ breast cancer cell lines and their expression of *neu*. Amplification and overexpression of neu are known to enhance cellular transformation and increase the metastatic potential of cancer cells. Clinically, they are also correlated with more aggressive tumor phenotypes. Therefore, expression of neu may represent a key regulatory point in estrogenic control of cellular growth and transformation. In this communication we demonstrate that the presence of E2/ER can repress transformation of NIH/3T3 cells by the neu oncogene. Furthermore, we have investigated the effects of E2/ER on growth and transformation of an ER+, neu-overexpressing breast cancer cell line. We report that the presence of E2/ER in these cells leads to regression of the transformed phenotype (as measured by anchorage-independent growth) while stimulating cellular proliferation (in monolayer culture) and propose a model for the role of neu in progression to hormone independence based on these results.

Currently, we are tirating the ratio between TAM and E2 ratio to find the optimum condition for antagonism of TAM to the E2-mechated effects.

4. Effects of Rb on Malignant Transformation Phenotypes of HER-2/*neu*-overexpressing Breast Cancer Cells

In the last Progress Report, we have identified MDA-MB-468 and SK-OV-3 cell lines expressing aberrant form of Rb protein. We also co-transfected Rb expressing vector and pSV2-neo plasmid (10:1 molar ratio between Rb and neo plasmid) into SK-OV-3 cells. We selected 20 G418 resistant colonies (The pSV2-neo can convert the recipient cells to become G418-resistant) and screened for wt. Rb expression. None of these 20 colonies express wt Rb protein. This may be due to the cell growth arrest property of Rb protein. It is known in the tumor suppressor field that stable transfectants of tumor suppressor genes (such as Rb or p53) are difficult to obtain as expression of these tumor suppressor genes frequently suppress cell growth and can not grow to a colony to be selected. To overcome this problem, we have constructed adenovirus-Rb expressing vector (AdV.Rb). This way, we can infect cancer cells with adenovirus-Rb vector which allows transient expression of Rb gene in the infected cells and allows us to observe the effect of biological property. We also characterized more breast cancer cell lines regarding to Rb and HER-2/neu status. Three cell lines have been tested. They are SKBr3 (wt, Rb, HER-2/neu overexpression), BT549 (mut Rb, HER-2/neu basal level) and MDA-MB-468 (mut Rb, HER-2/neu basal level). The effects of Rb on cell growth rate (measured by MTT assay) and in vitro transformation phenotype (measured by soft aganose colonization assay) were shown in Figs. 4-6. There is no significant effect on cell growth based on MTT assay after AdVRb infection to breast cancer cells as shown in Panel A of Figs 4-6. However, the AdVRB infection significantly suppress soft aganose colonization activity in the two Rb mut lines. MDA-MB-468 (Fig. 5) and BT-549 (Fig. 4), indicating that wt Rb indeed can suppress transformation phenotype of Rb-defective cancer cells. Interestingly AdVRb has no significant effect on SkBr3 in which HER-2/neu is overexpressed and Rb is wt. It is not yet clear whether HER-2/neu expression in SkBr3 is repressed by the AdVRb. Further study is required to clarify the relationship between effect of AdVRb on these breast cancer cell lines.

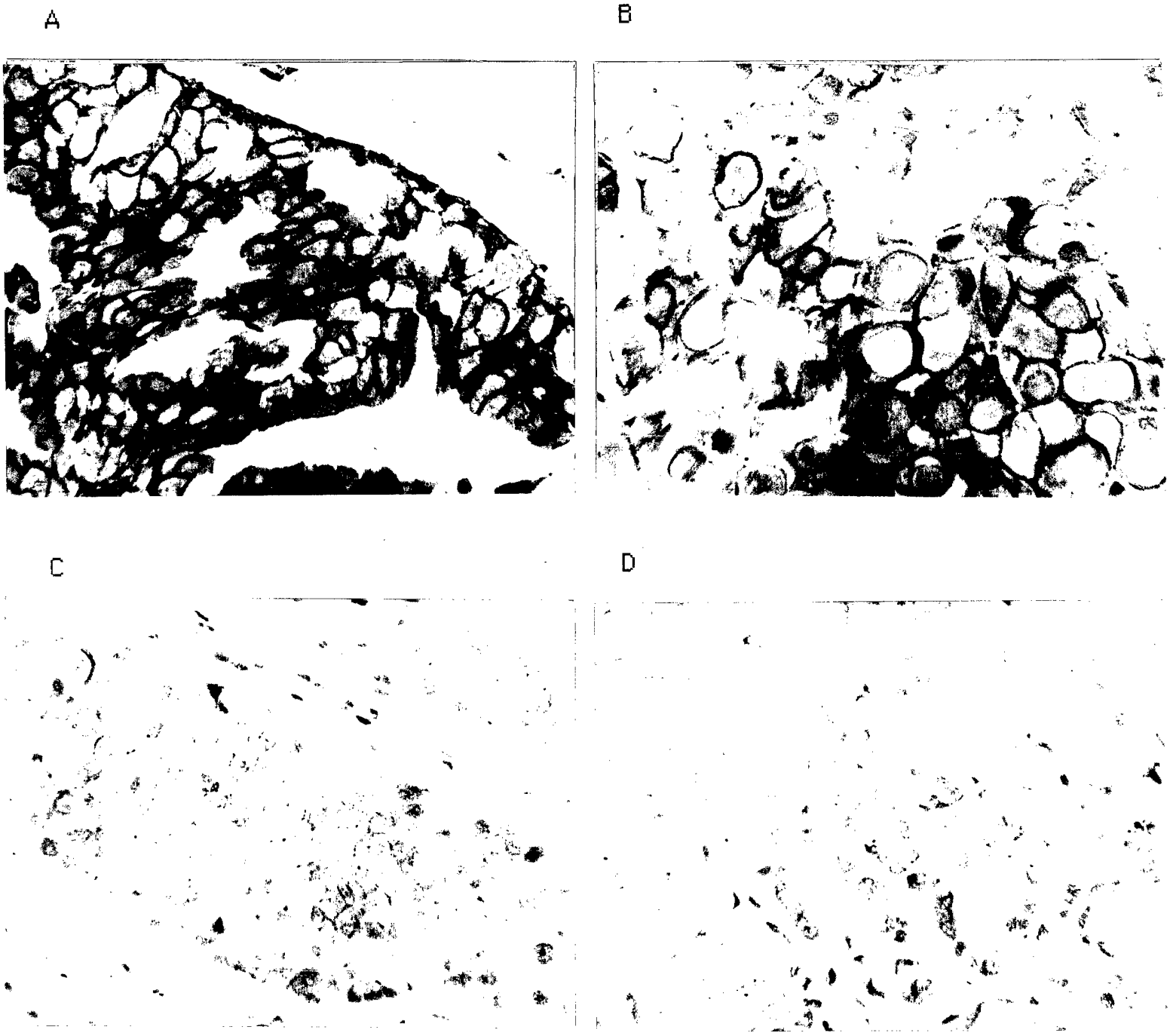

Fig. 1. Immunohistochemical staining of breast cancer with EGFR antibody. (A) High(+++), (B) Intermediate(++), (C) Low(+), And No expression(-). Magnification: X400

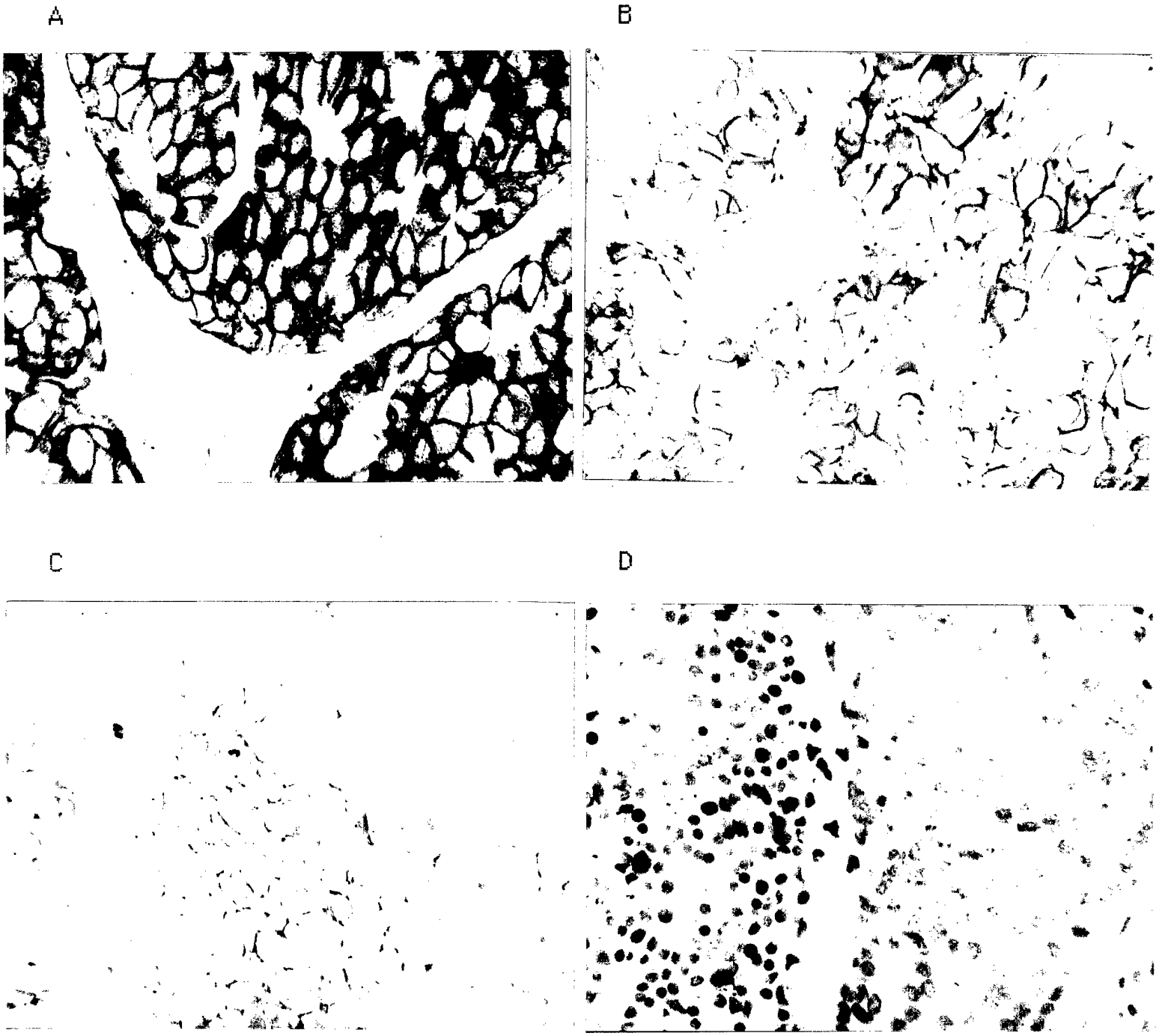

Fig. 2. Immunohistochemical staining of breast cancer with HER-2/neu antibody. (A) High(+++), (B) Intermediate(++), (C) Low(+), And No expression(-). Magnification: X400

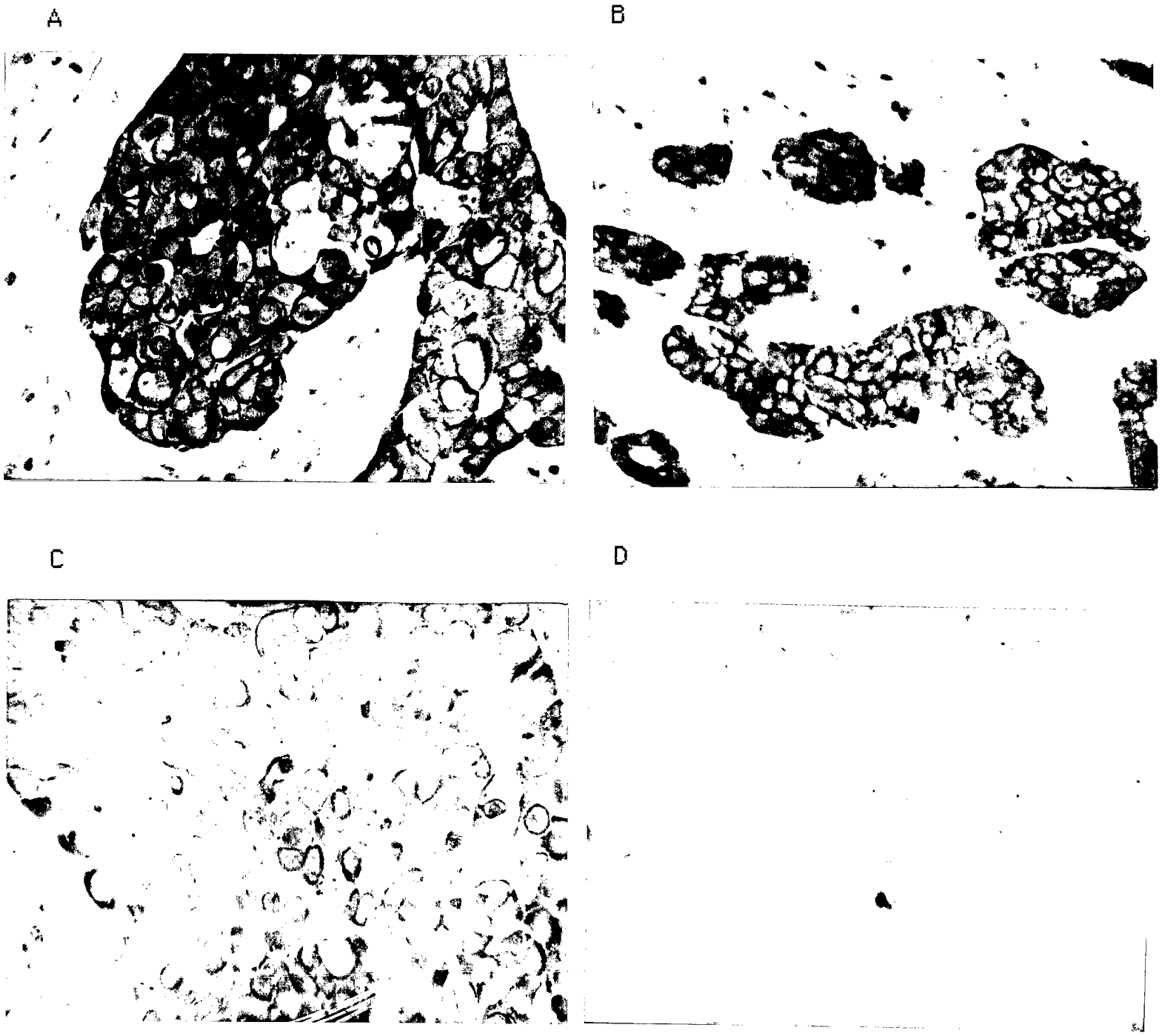

Fig. 3. Immunohistochemical staining of breast cancer with HER-3 antibody. (A) High(+++), (B) Intermediate(++), (C) Low(+), And No expression(-). Magnification: X400

TABLE 1.

MB ADJUVANT STUDIES CROSS TABULATION Sep 04, 1996 10:34:45 PAGE 1
FROM SEARCH TEMPLATE LAHONG HER-2NEU

CHARACTERISTICS OF 123 CASES

	HER-2NEU NEG	1	2	3	ROW TOT
TOTAL :	67	16	9	31	123
AGE GROUP:					
AGE <50	57	13	8	23	101
AGE =>50	10	3	1	8	22
RACE:					
WHITE	46	10	7	18	81
BLACK	7	1	1	2	11
HISPANIC	9	4	1	8	22
OTHER	3	1	-	3	7
Missing	2	-	-	-	2
MENOPAUSAL STATUS:					
PRE	44	9	8	15	76
PERI	3	1	-	4	8
POST	11	2	1	10	24
SURG	9	4	-	2	15
RSTAGE (NUMERIC):					
1	7	2	-	2	11
2	53	11	6	27	97
3	5	2	3	-	10
4	2	1	-	2	5
RNODES:					
Missing	-	1	-	-	1
N=0	22	4	1	9	36
N=1-3	33	6	5	15	59
N=4-10	9	4	1	4	18
N>10	3	1	2	3	9
ER:					
NEG	40	10	1	17	68
POS	23	6	7	11	47
UNK	4	-	1	3	8
STATUS:					
ALIVE/NED	52	10	7	26	95
ALIVE/WD	11	2	1	2	16
DEAD/NED	1	-	-	1	2
DEAD/WD	3	4	1	2	10
TUMOR GRADE (NUCLEAR):					
NG1	43	7	3	17	70
NG2	20	9	5	13	47
UNK	4	-	1	1	6

RRECUR:					
NO	53	10	7	27	97
YES	14	6	2	4	26
RXRT:					
NO	43	10	5	19	77
YES	24	6	4	12	46
FIRST SITE/1ST RECUR:					
BONE	6	2	-	-	8
LUNG	2	-	-	1	3
NODES	1	1	1	2	5
LIVER	-	1	-	1	2
CW	3	-	-	-	3
SKIN	-	2	-	-	2
OP BREAST	1	-	-	-	1
INT-ABD	-	-	1	-	1
SOFT TISSUE	1	-	-	-	1
Missing	53	10	7	27	97
RTSIZE:					
1	47	7	4	22	80
2	18	6	3	9	36
3	2	3	2	-	7
SURG TYPE:					
RAD	1	1	-	-	2
MOD RAD	50	15	7	27	99
LUMPECT	3	-	-	-	3
SEG/AX D	13	-	2	4	19
PROT:					
82-79	1	-	-	-	1
86-12	66	16	9	31	122

Figure 4a. Cell Growth After Infection with AdVRb as Measured by MTT Assay

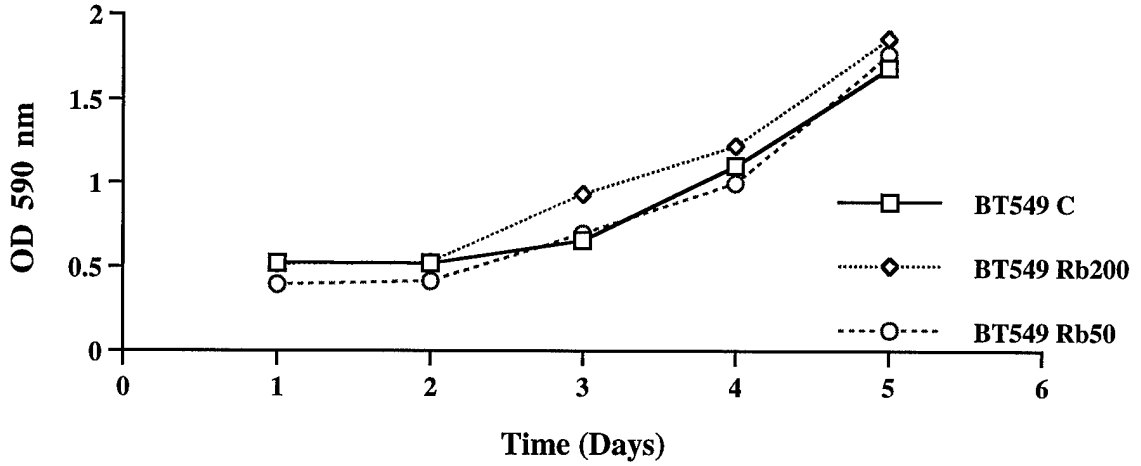

Figure 4b. Colony Formation in Soft Agarose After Infection with AdVRb

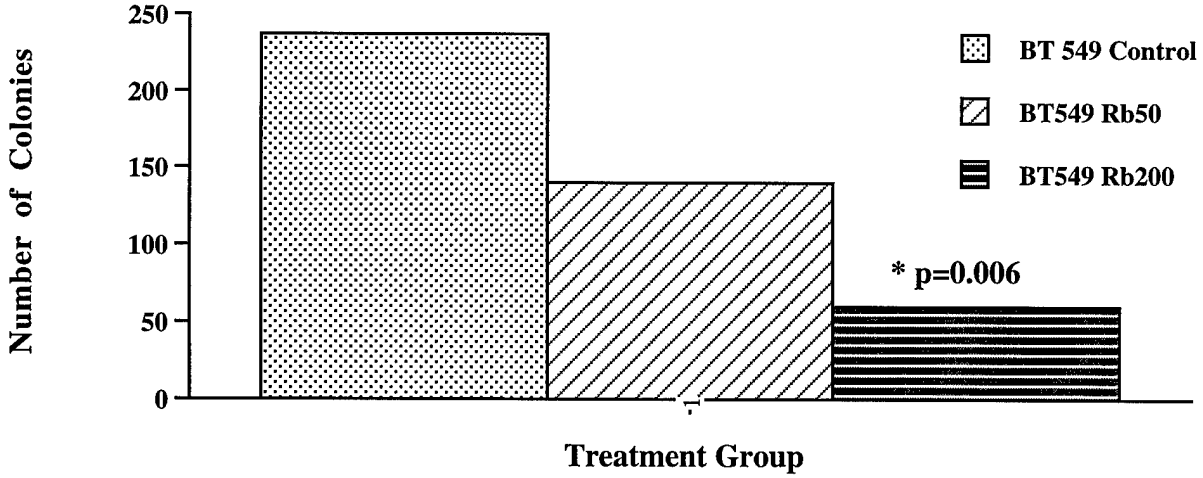

Figure 4. Effect of AdVRb on breast carcinoma cell line BT549 (Rb-null cell line).

Figure 4. Effect of AdVRb on breast carcinoma cell line BT549 (Rb-null cell line). The cell line was maintained under standard cell culture conditions. Treatment groups included i) untreated cells (BT549 C and BT549 control), ii) cells infected with the recombinant adenovirus vector containing a gene coding for an N-terminal truncated retinoblastoma (Rb) gene product (AdVRb) at a multiplicity of infection (MOI) of 200 virus particles to each breast cancer cell (BT549 Rb200), and iii) cells infected with AdVRb at a multiplicity of infection of 50 virus particles to each breast cancer cell. **Figure 4a.** Cell growth after infection with AdVRb as measured by MTT assay. Two days after treatment with AdVRb, cells were trypsinized and counted and then seeded onto 96-well plates at a density of 1,000 cells per well. Cell growth was then evaluated by measuring the metabolically active cells with MTT {MTT=(3,(4,5,-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide)} staining at 24 hour intervals for five consecutive days. Optical density was determined at a wavelength of 590 nm. No significant difference was observed in the growth curves of untreated cells versus those treated with AdVRb at an MOI of 200:1 or 50:1. **Figure 4b.** Colony formation in soft agarose after infection with AdVRb. Two days after treatment with AdVRb, cells were trypsinized and counted and then mixed with 0.7% agarose in 1XDMEM with 10% fetal calf serum. This cell/agarose mixture was layered onto a 1.4% agarose base in 12-well plates. Cells were incubated at 37°C for three weeks. The number of colonies forming in this time period were counted and scored (measurement of anchorage independent growth). There was a significant decrease in the number of colonies which developed in the control group versus the cells treated with AdVRb at an MOI of 200:1 ($p=0.006$, two-tailed student's t-test).

Figure 5a. Cell Growth After Infection with AdVRb as measured by MTT Assay

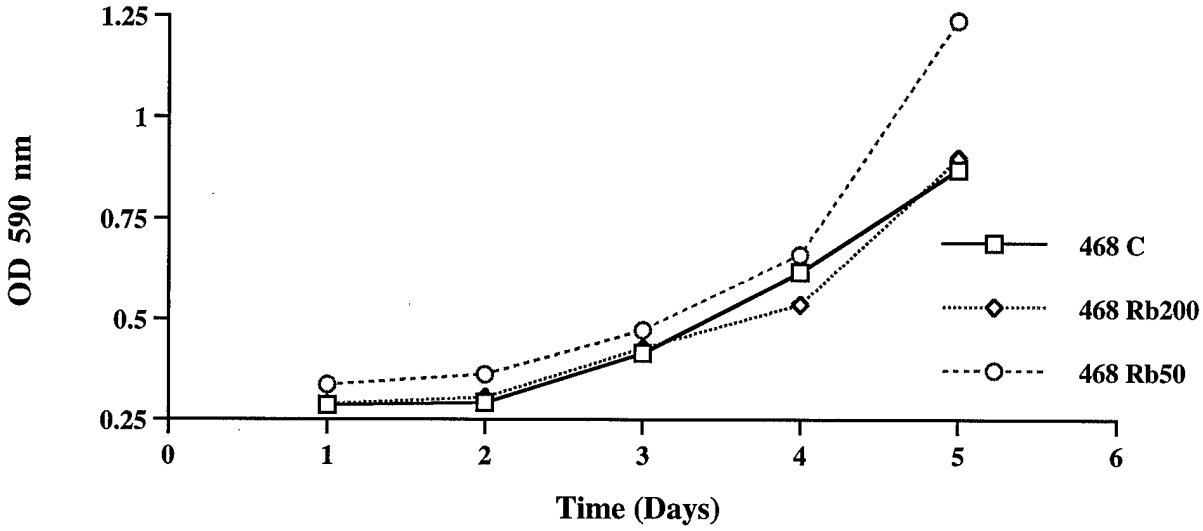

Figure 5b. Colony Formation in Soft Agarose After Infection with AdVRb

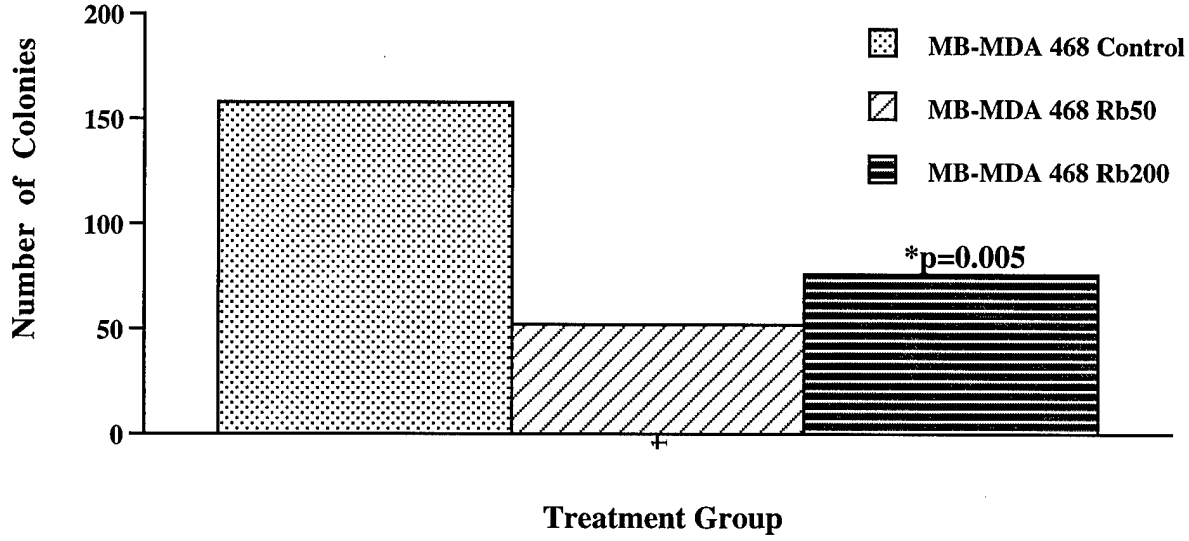

Figure 5. Effect of AdVRb on breast carcinoma cell line MB-MDA-468 (Rb-null cell line).

Figure 5. Effect of AdVRb on breast carcinoma cell line MB-MDA-468 (Rb-null cell line). The cell line was maintained under standard cell culture conditions. Treatment groups included i) untreated cells (468 C and MB-MDA 468 control), ii) cells infected with the recombinant adenovirus vector containing a gene coding for an N-terminal truncated retinoblastoma (Rb) gene product (AdVRb) at a multiplicity of infection (MOI) of 200 virus particles to each breast cancer cell (468 Rb200 and MB-MDA 468 Rb200), and iii) cells infected with AdVRb at a multiplicity of infection of 50 virus particles to each breast cancer cell (468 Rb50 and MB-MDA 468 Rb50). **Figure 5a.** Cell growth after infection with AdVRb as measured by MTT assay. Two days after treatment with AdVRb, cells were trypsinized and counted and then seeded onto 96-well plates at a density of 1,000 cells per well. Cell growth was then evaluated by measuring the metabolically active cells with MTT {MTT=(3,(4,5,-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide)} staining at 24 hour intervals for five consecutive days. Optical density was determined at a wavelength of 590 nm. No significant difference was observed in the growth curves of untreated cells versus those treated with AdVRb at an MOI of 200:1 or 50:1. **Figure 5b.** Colony formation in soft agarose after infection with AdVRb. Two days after treatment with AdVRb, cells were trypsinized and counted and then mixed with 0.7% agarose in 1XDMEM with 10% fetal calf serum. This cell/agarose mixture was layered onto a 1.4% agarose base in 12-well plates. Cells were incubated at 37°C for three weeks. The number of colonies forming in this time period were counted and scored (measurement of anchorage independent growth). There was a significant decrease in the number of colonies which developed in the control group versus the cells treated with AdVRb at an MOI of 200:1 ($p=0.005$, two-tailed student's t-test).

Figure 6a. Cell Growth After Infection with AdVRb as Measured by MTT Assay

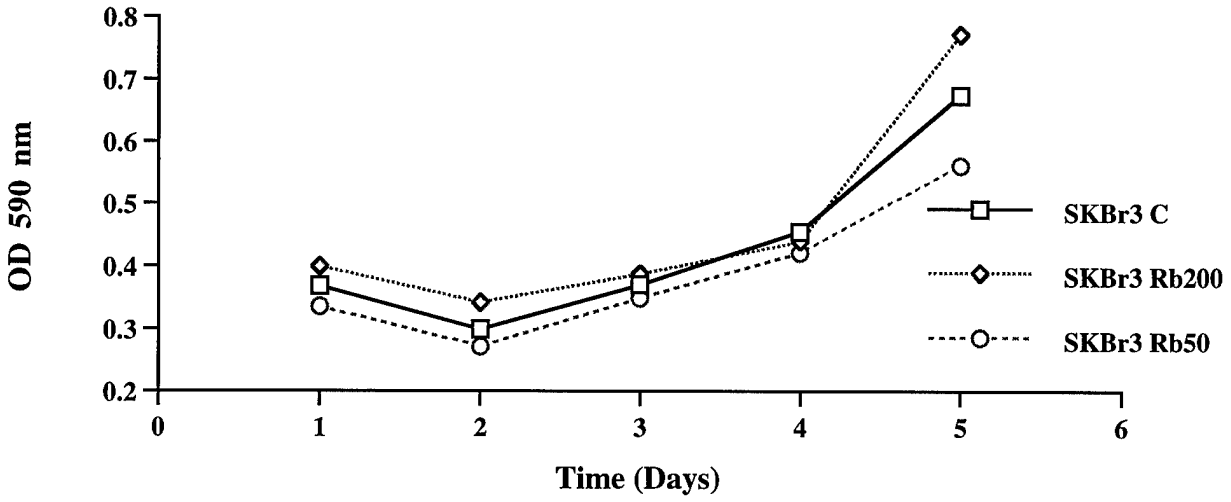

Figure 6b. Colony Formation in Soft Agarose After Infection with AdVRb

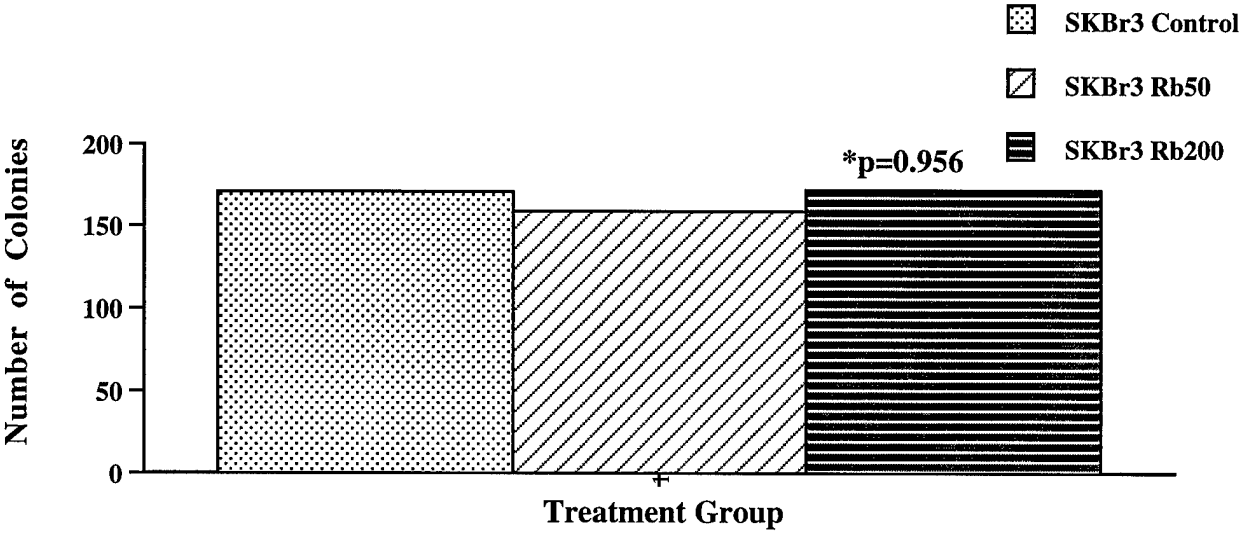

Figure 6. Effect of AdVRb on breast carcinoma cell line SKBr3 (Rb-wild-type cell line).

Figure 6. Effect of AdVRb on breast carcinoma cell line SKBr3 (Rb-wild-type cell line). The cell line was maintained under standard cell culture conditions. Treatment groups included i) untreated cells (SKBr3 C andSKBr3 control), ii) cells infected with the recombinant adenovirus vector containing a gene coding for an N-terminal truncated retinoblastoma (Rb) gene product (AdVRb) at a multiplicity of infection (MOI) of 200 virus particles to each breast cancer cell (SKBr3 Rb200), and iii) cells infected with AdVRb at a multiplicity of infection of 50 virus particles to each breast cancer cell (SKBr3 Rb50). **Figure 6a.** Cell growth after infection with AdVRb as measured by MTT assay. Two days after treatment with AdVRb, cells were trypsinized and counted and then seeded onto 96-well plates at a density of 1,000 cells per well. Cell growth was then evaluated by measuring the metabolically active cells with MTT {MTT=(3,(4,5,-dimethylthiazol-2-yl) 2,5 diphenyl tetrazolium bromide)} staining at 24 hour intervals for five consecutive days. Optical density was determined at a wavelength of 590 nm. No significant difference was observed in the growth curves of untreated cells versus those treated with AdVRb at an MOI of 200:1 or 50:1. **Figure 6b.** Colony formation in soft agarose after infection with AdVRb. Two days after treatment with AdVRb, cells were trypsinized and counted and then mixed with 0.7% agarose in 1XDMEM with 10% fetal calf serum. This cell/agarose mixture was layered onto a 1.4% agarose base in 12-well plates. Cells were incubated at 37°C for three weeks. The number of colonies forming in this time period were counted and scored (measurement of anchorage independent growth). There was no significant difference in the number of colonies which developed in the control group versus the cells treated with AdVRb at an MOI of 200:1 ($p=0.956$, two-tailed student's t-test).

CONCLUSION:

Task 1: Screening of 200 tumor sections for EGF receptor, HER-2/*neu* and HER-3 has been completed. Due to the complexity caused by the newly identified HER-3 and HER-4 (12, 13), we will emphasize more on the staining of EGF receptor, HER-2/*neu*, HER-3, HER-4 and Heregulin. We also have begun to collect data and analyze between gene expression and clinical status from medical record.

Task 2: Construction of Heregulin-expression vectors and anti-sense Heregulin plasmids have been completed. Stable transfectants of both Heregulin and anti-sense Heregulin are completed. Characterization of these stable transfectants is currently underway.

Task 3: Characterization of E2 on ER$^+$, HER-2/*neu*-overexpressing breast cancer cells was completed. A manuscript describing these works was published this year. Characterization of antagonism of Tamoxifen (TAM) to estrogen (E2)-mediated effect has been started.

Task 4: Screening of Rb expression in breast cancer cell lines was completed. We will focus on SKBr3, BT549 and MDA-MB-468 as described in the Progress Report (Figs 4-6). Stable transfectants of SK-OV-3 did not result in Rb-expressing stable transfectants. We have used another approach to study the effect of Rb on breast cancer cells by construction of adenovirus Rb expressing vector (AdV.Rb). some biological characterizations were presented in Figs 4-6. To our surprise, AdV.Rb has no effect on SkBr3. We will further characterize the biological effects and expression of Rb and HER-2/*neu* after AdV.Rb infection.

In addition to the those described in the BODY Section, several studies relating to HER-2/neu oncogene in breast cancer have been completed. The funding support from the current project has been appropriately acknowledged in the resulting publications. These include:

1. Kerry Strong Russell, Eun Kyung Lee, Nobutaka Kiyokawa, Saya Hideyuki and Mien-Chie Hung. Effects of estrogen receptor expression on growth and transformation of cells overexpressing *neu*. *Oncology Reports* 3:433-437, 1996.

2. Youming Xie and Mien-Chie Hung. p66^{Shc} isoform down-regulated and not required for HER-2/*neu* signaling pathway in human breast cancer cell lines with HER-2/*neu* overexpression. *Biochem. Biophys. Res. Comm.* 221:140-145, 1996.

3. Youming Xie, Ann Marie Pendergast and Mien-Chie Hung. Dominant-negative mutants of GRB2 induced reversal of the transformed phenotypes caused by the point mutation-activated rat HER-2/*neu*. *J. Biol. Chem.* 270(51):30717-30724, 1995.

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5. Xiangming Xing, Angabin Matin, Dihua Yu, Weiya Xia, Frank Sorgi, Leaf Huang and Mien-Chie Hung. Mutant SV40 large T antigen as a therapeutic agent for HER-2/*neu*-overexpressing ovarian cancer. *Cancer Gene Therapy* 3(3):168-174, 1996.

Reprints or copies of these papers are attached.

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Effects of estrogen receptor expression on growth and transformation of cells overexpressing *neu*

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Abstract. The mechanism by which breast cancers progress to hormone independence does not always require the loss of estrogen receptor(ER) expression or function. Cellular alterations that disturb the normal pathway of estrogen-regulated growth may contribute to a state of hormone independence. We and others have described an inverse relationship between estrogen stimulation of ER$^+$ breast cancer cell lines and their expression of *neu*. Amplification and overexpression of *neu* are known to enhance cellular transformation and increase the metastatic potential of cancer cells. Clinically, they are also correlated with more aggressive tumor phenotypes. Therefore, expression of *neu* may represent a key regulatory point in estrogenic control of cellular growth and transformation. In this communication we demonstrate that the presence of E2/ER can repress transformation of NIH/3T3 cells by the *neu* oncogene. Furthermore, we have investigated the effects of E2/ER on growth and transformation of an ER$^+$, *neu*-overexpressing breast cancer cell line. We report that the presence of E2/ER in these cells leads to repression of the transformed phenotype (as measured by anchorage-independent growth) while stimulating cellular proliferation (in monolayer culture) and propose a model for the role of *neu* in progression to hormone independence based on these results.

Introduction

Proto-oncogene *neu* (also known as HER-2 or c-*erb*B2) encodes a receptor protein kinase, overexpression of which is correlated with enhanced malignancy and poor clinical outcome (1-5). Conversely, repression of *neu* leads to loss of the transformation phenotype (4,6-8). Clinical correlations have shown *neu* to be an important prognostic factor in breast cancer (1-3). Another prognostic factor is estrogen receptor (ER) status (9-11). Estrogen is known to stimulate cell proliferation, and antiestrogen, such as tamoxifen, is used in ER$^+$ breast cancer patients to suppress estradiol (E2)-stimulated cell growth. Several studies have shown that there exists an inverse correlation between expressions of *neu* and ER (12,13). Clinical studies have shown that coexpression of *neu* and ER is correlated with tamoxifen resistance (14,15). These studies together suggest that there is an interaction between the *neu* gene product and ER. One recent report suggested that activation of *neu* leads to phosphorylation of ER on tyrosine residues and subsequent activation of estrogen-responsive genes (16). On the other hand, we and others have previously shown that estrogen-stimulated ER (E2/ER) can repress expression of *neu* (17,18) at the transcriptional level (19). These studies together suggest a possible feedback loop between ER and *neu*. While the ER/*neu* regulatory mechanism has been studied, the question of whether repression of *neu* by E2/ER plays a physiologically important role in suppressing transformation by this oncogene has not been addressed yet. Our hypothesis is that E2 has dual effects on ER$^+$, *neu*-overexpressing cancer cells; while it stimulates cell growth, E2 at the same time modulates *neu* expression and thereby inhibits transformation in these cells. In this study, using our previously established ER$^+$, *neu*-overexpressing cell lines, we tested this hypothesis.

Materials and methods

Cell culture. The human breast cancer cell line BT-474 was obtained from the American Type Culture Collection (Rockville, MD). This line was routinely maintained in phenol red-free RPMI supplemented with 10% fetal bovine serum. NIH/3T3 cells were maintained in phenol red free Dulbecco's Modified Eagle's Medium (DMEM). To remove steroid hormone agonists present in normal serum, the serum used in these experiments was treated by incubation with dextran-coated charcoal (20). The final concentration of estrogen in serum thus treated was measured by radioimmunoassay (ICN Biomedicals, Lisle, IL) and found to be $\leq 10^{-12}$ M. Stock solutions of E2 was dissolved

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Abbreviations: ER, estrogen receptor; ER$^+$, estrogen receptor positive; E2/ER, estrogen-stimulated estrogen receptor; ER$^-$, estrogen receptor negative; E2, estradiol; *neu**, activated *neu*

Key words: estrogen receptor, HER-2, breast cancer

in ethanol at concentrations of 0.1-1 mM and stored at -20°C for periods of up to one month. The concentration of ethanol in all plates, both steroid treated and deprived, was kept at 0.01%.

Focus formation assays. NIH/3T3 fibroblasts were transfected in regular DMEM/F12 plus 10% calf serum by calcium phosphate-mediated gene transfer (21). The cells were washed with phosphate-buffered saline (PBS) after 12-16 h of treatment with the DNA-$CaPO_4$ precipitate and fed with 10 ml of regular medium. The next morning, each plate was split 1:4. Two of these plates were fed with estrogen-depleted medium plus 10 nM E2, and the other two with estrogen-depleted medium plus ethanol (to bring the final concentration of ethanol in all plates to 0.01%). Plates were refed with the same medium every other day for 3-4 weeks. The plates were then washed with PBS and stained with 1% crystal violet (Sigma) in 20% ethanol. Foci of stained cells >1 mm in diameter were scored manually by using a 1-cm grid.

Formation of ER+ sublines. Stable sublines of BT-474 cells expressing ER were formed by using a viral infection scheme as described previously (19). Subclones denoted '(i/ER)' were infected with the PXTHER viral vector containing 2.1 kb of human ER cDNA along with the neomycin resistance gene (neo^R), and those denoted '(i/neo)' were infected with the PXTI vector containing neo^R alone (22). Stable cell lines resistant to neomycin were subsequently subcloned and tested for expression of ER. We have previously demonstrated that the PXTHER-containing subclones B1(i/ER) and B10(i/ER) express ER mRNA, while the control PXTI-containing subclone B1(i/neo) does not express ER mRNA (19).

Soft agar assays. Plates (60 mm) were prepared by placing a 2 ml bottom layer of estrogen-depleted medium and 0.7% agarose (SeaPlaque) ± 10 nM E2. Cells were harvested from subconfluent plates by trypsinization. After the bottom layer had solidified, $3x10^4$ cells were plated (in triplicate) in 2 ml of estrogen-depleted medium and 0.35% agarose ±E2 as a top layer. Plates were fed with 3 drops of medium every other day. After 3-4 weeks, colonies were stained by placing 1 ml of INT (p-iodonitrotetrazolium violet, 1 mg/ml) on each plate and incubating overnight at 37°C. Colonies of violet-stained (viable) cells were counted manually using a 5.0-mm grid.

Growth curves in monolayer culture. For each cell line, 10^4 cells/well were seeded into 6-well plates on day 0 in estrogen-depleted medium ± 10 nM E2. To those plates without added E2, ethanol was added such that its final concentration in all plates, both steroid treated or depleted, was kept at 0.01%. Medium was changed at 3-day intervals, again with or without E2. At various timepoints after plating, duplicate wells were harvested by trypsinization and the cells counted by an automated system (Coulter Instruments).

Results

The presence of E2/ER affects the ability of neu to transform NIH/3T3 cells. To test the hypothesis that regulation of *neu* expression by E2/ER can prevent cellular transformation by *neu*, a focus formation system was utilized. NIH/3T3 cells were transfected with DNA encoding transforming oncogene *neu*. In each plate, only a limited percentage of these cells received and expressed the oncogene. The cells that were transformed by the oncogene piled up as they proliferated to form foci of transformed cells in a background of non-transformed NIH/3T3 cells. These foci were easily visualized and counted after staining. The number of foci formed is a function of the efficiency of gene transfer as well as the efficiency of expression of the oncogene-encoded product. In later experiments, differences in gene transfer and maintenance were eliminated by comparing only plates of cells from a single transfection. This was achieved by splitting a single transfected plate into four identical plates for subsequent treatment with E2 (Table I).

Table I. Focus formation by *neu** and *ras* transfected NIH/3T3 cells.

	Avg #foci (n=2)[a]	
Oncogene vector	+E2/ER	-E2/ER
c-*nu* 104 (*neu**)	33	82
	24	79
pT24-C3 (*ras*)	57	55
	70	65

[a]Note that each row represents a single transfection, therefore data can only be compared across single rows. Direct comparisons between rows are invalid since transfection efficiency may alter the number of cells receiving the transforming vector. These data were normalized to form Fig. 1 by measuring repression by estradiol in each transfection and comparing these values.

NIH/3T3 cells were transfected with the c-*nu*-104 expression construct, which contains the transforming rat *neu** (activated *neu*) oncogene under the control of its endogenous promoter (23). Table I shows that the presence of E2 and ER dramatically reduced the ability of c-*nu*-104 to induce focus formation in NIH/3T3 cells. This result is consistent with our previous data suggesting that E2/ER can reduce transcription from the *neu* promoter and thus expression of $p185^{neu}$.

In order to assess whether inhibition of NIH/3T3 transformation by E2/ER is a generalized phenomenon, these experiments were repeated using another transforming oncogene. The pT24-C3 construct contains a 6.4 kb portion of the human homolog to the H-*ras* oncogene. The gene is under control of its own promoter in this construct and has been shown to be a potent transforming oncogene in focus formation assays (24). When this construct was co-transfected with plasmid expressing estrogen receptor into NIH/3T3 cells, no difference in focus formation was seen between cells grown in the presence of E2 and those grown in the absence of E2 (Fig. 1). Therefore, the inhibition of *neu** mediated transformation of NIH3T3 cells by E2/ER is oncogene specific, in that E2/ER does not alter transformation by *ras*.

Estradiol inhibits the ability of BT-474 (i/ER) sublines to grow in soft agar. The previous findings suggest that, by

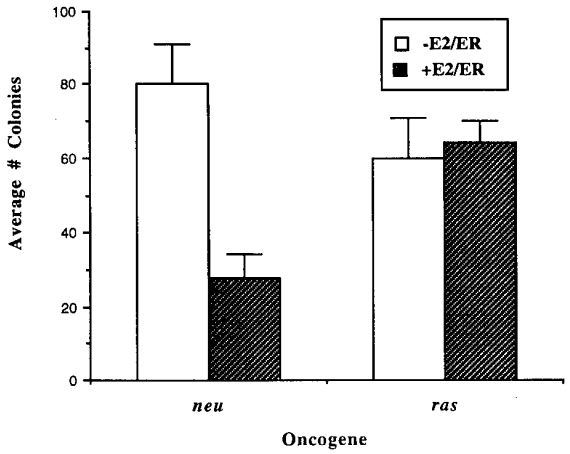

Figure 1. Focus formation by *neu*⁺-and *ras*-transfected NIH/3T3 cells. NIH/3T3 fibroblasts were transfected with either *neu*⁺ or *ras* with or without ER. Plates from a single transfection were then split 1:4 and plated in the presence or absence of 10 nM E2. Foci of transformed NIH/3T3 cells were counted after 3-4 weeks of culture.

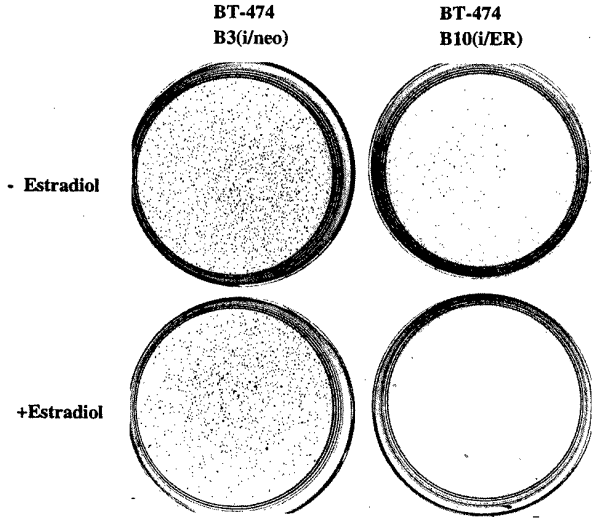

Figure 2. Soft agar colony formation by BT-474 subclones. Subconfluent plates of each subclone were harvested and plated in agarose ± 10 nM E2. Colonies were stained and counted manually after 3-4 weeks of culture. Data are summarized in Fig. 3.

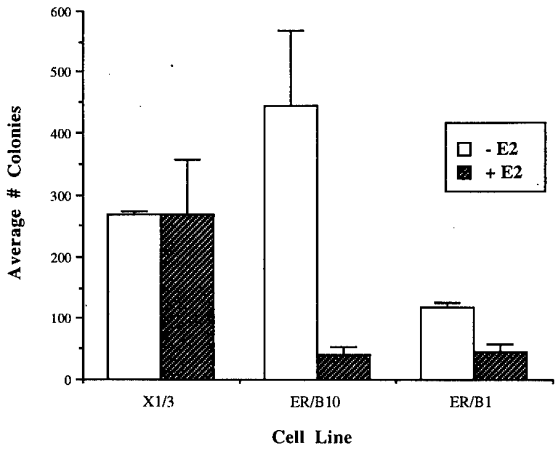

Figure 3. Summary graph of soft agar colony formation by BT-474 subclones.

Table II. Doubling times of BT-474 subclones in monolayer culture.

BT474 subclone	Doubling time (days) +Estradiol	Doubling time (days) -Estradiol	p
X1/3(i/neo)	3.81	3.91	n.s.
B10(i/ER)	3.24	4.70	<0.001
B1(i/ER)	2.89	4.43	<0.02

n.s., not significant.

repressing *neu* expression, E2 and ER may play a role in regulating the ability of *neu* to act as a transforming oncogene. To test this possibility in human breast cancer cell lines, the BT-474 breast cancer cell line, which is ER⁻ and *neu*-overexpressing (25), and its ER transfectants were chosen for these experiments. Although there is a report indicating that a BT-474 subline expressed a low level of ER (26), Northern blot analysis of our BT-474 line for ER mRNA expression established that our BT-474 cell line does not express detectable amount of ER and does not respond to E2 (19). The formation of ER-expressing subclones of the BT-474 human breast cancer cell line was previously described (19). When ER⁺ (i/ER) and ER⁻ (i/neo) subclones were tested for their ability to grow in an anchorage-independent manner, we found that the ability of the (i/ER) subclones to form colonies in soft agar was significantly reduced in the presence of E2 (Figs. 2 and 3). E2 had no effect on the ability of the ER⁻ (i/neo) subclone to form colonies in these experiments, which agrees with the idea that E2 alone cannot repress the expression of *neu* in these cells (19). These results clearly indicate that E2 can suppress transformation of ER⁺, *neu*-overexpressing cancer cells but has no effect on ER⁻, *neu*-overexpressing cells. This suppression is most likely caused by E2/ER-mediated *neu* repression.

Estradiol stimulates the ability of BT-474 (i/ER) sublines to grow in monolayer culture. The results presented above agree with data showing a correlation between increased *neu* expression and cellular transformation (in this case, measured by anchorage-independent growth) (27). The addition of E2/ER to these cells both suppresses *neu* expression and leads to a reduction in their anchorage-independent growth. It is important to note that in other ER⁺ breast cancer cell lines [e.g., MCF-7 (28) and ZR-75-1 (29)], E2 stimulates growth in monolayer cultures. Since grdwth in soft agar and in monolayer culture are not necessarily correlated, growth curves were constructed to assess the effects of E2 and ER on the growth of the BT-474 subclones in monolayer culture.

Table II shows the results from these experiments. Growth of the (i/ER) subclones was significantly enhanced in the presence of E2, whereas E2 had no effect on the growth of the ER⁻ control subclone (X1/3). The presence of ER in these clones significantly altered their growth characteristics, such

that doubling times for the (i/ER) clones decreased in the presence of E2, but were higher in estrogen-depleted medium than those of the X1/3(i/neo) ER$^-$ clone. Thus, the presence of ER in these cells allows E2-stimulated growth in monolayer culture. The results shown here demonstrate that E2 indeed has dual effects on ER$^+$, *neu*-overexpressing cancer cells; E2 will enhance transformation activity (measured by anchorage independence) but inhibit cell growth rate (measured by growth in monolayer).

Discussion

In this report, we have shown that E2/ER indeed has dual effects on ER$^+$, *neu*-overexpressing cancer cells-stimulation of cell growth while suppressing transformation via *neu* repression. This further confirms the importance of the interaction between E2/ER and *neu* in cancer cells. Coexpression of ER and *neu* has been studied previously at the experimental (26) and clinical levels (14,15). It has been reported that coexpression of *neu* in ER$^+$ MCF-7 cells leads to increased tumorigenesis (26). Our results show that coexpression of ER in *neu*-overexpressing BT474 cells leads to a decrease in transformation upon E2 stimulation. Our data do not contradict the previous report since we are proposing that E2/ER suppression of transformation is due to *neu* repression at the promoter level. The authors of the previous report (26) used ER$^+$ MCF-7 cells transfected with a full-length cDNA encoding *neu* under exogenous promoter control. In such a system, E2/ER cannot inhibit *neu* expression; yet, E2 can still stimulate cell growth through the ER pathway and thereby promote tumorigenesis.

Based on our studies, the following model for transformation/progression to hormone independence of the BT-474 cell lines is proposed. In these cells, loss of ER expression results in derangement of pathways normally regulated by this receptor. The growth of these cells is no longer stimulated in the presence of E2 (Table II). Another effect of deregulation of ER pathways is the de-repression of *neu* expression and consequent increase in cellular transformation (as demonstrated by ability to grow in soft agar). Finally, restoring the expression of ER reconstitutes these pathways such that stimulation with E2 increases cellular growth rate (in monolayer culture) and reduces the level of *neu* expression in response to E2 stimulation so that cells are less transformed.

This model leads to an interesting hypothesis regarding tamoxifen resistance. The mechanism of tamoxifen resistance in breast cancer is an area of intensive research. Tamoxifen is the endocrine therapy of choice for ER$^+$ breast cancer patients and is used to suppress cell growth. Tamoxifen, however, is effective in only about 50-60% of ER$^+$ patients (14,15,30,31). Recent clinical studies have shown that coexpression of *neu* in ER$^+$ breast cancer patients is correlated with decreased response to tamoxifen treatment, compared with those ER$^+$ patients without overexpression of *neu* (14,15). An experimental study has shown that coexpression of *neu* in ER$^+$ MCF7 cells leads to increased tamoxifen resistance (26). In this case, the exogenous *neu* expressing vector is not driven by the *neu* promoter and therefore *neu* expression will not be affected by tamoxifen. However, it has been shown that tamoxifen can up-regulate *neu* expresison in ER$^+$ breast cancer cell lines (32). Taken together with our current study, we hypothesize that the tamoxifen-induced *neu* expression may be mediated by blocking the E2/ER-induced down-regulation of *neu* and that it contributes to the decreased response to tamoxifen for patients with ER$^+$, *neu*-overexpressing breast cancer. Based on this hypothesis, we speculate that treating ER$^+$, *neu*-overexpressing cells with an antiestrogen such as tamoxifen, though it may decrease cell proliferation, will increase transformation by disrupting normal E2 regulatory pathways involving *neu* repression. Clinically, when patients with ER$^+$, *neu*-overexpressing breast cancer are treated with tamoxifen, the antiproliferative effect of tamoxifen may be antagonized by tamoxifen's indirect effect of upregulating *neu* expression. Currently there are numerous hypotheses for tamoxifen resistance. Our results provide one mechanism which at the same time can explain an important clinical correlation: that ER$^+$ patients whose tumors overexpress *neu* may be resistant to tamoxifen.

Acknowledgments

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p66^{Shc} Isoform Down-Regulated and Not Required for HER-2/neu Signaling Pathway in Human Breast Cancer Cell Lines with *HER-2/neu* Overexpression

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The *HER2/neu* protooncogene encodes a transmembrane receptor tyrosine kinase of M_r 185 kDa (called p185) which is structurally and functionally homologous to the epidermal growth factor receptor. Shc proteins are important downstream signal transducers of receptor tyrosine kinases. We reported here a novel finding that p66^{Shc} was absent or nearly absent in p185-overexpressing breast cancer cells. This inverse correlation of p185 overexpression and p66^{Shc} expression is probably specific to breast cancer cells because this phenomenon was not observed in p185-overexpressing human ovarian, lung, or oral cancer cells, or mouse fibroblast cells. In contrast, the p52^{Shc} and p46^{Shc} isoforms were expressed at similar levels in both p185-overexpressing and p185 basal level breast cancer cell lines. Furthermore, tyrosine phosphorylation of p52^{Shc} and p46^{Shc} and subsequent formation of Shc/Grb2 complex were detected in breast cancer cells in which the p185 tyrosine kinase is activated, indicating that p66^{Shc} is not required for mediating the HER-2/neu signaling pathway in breast cancer cells.

The *HER2/neu* (also known as *erbB*-2) protooncogene encodes an M_r 185,000 transmembrane glycoprotein (called p185) with intrinsic tyrosine kinase activity homologous to the epidermal growth factor (EGF) receptor (1–4). It is well known that amplification and/or overexpression of *HER2/neu* correlates to approximately 30% of human breast cancer (5) and that aberrant activation of its tyrosine kinase confers transforming ability on p185 (6). However, the downstream signaling pathways mediating the oncogenic signal of p185 in breast cancer are not well understood. Our recent studies have demonstrated that tyrosine phosphorylation of Shc, termed for *S*rc *h*omology 2α-collagen-related (7), and subsequent formation of the Shc/Grb2/Sos complex play a major role in cell transformation mediated by the point mutation-activated p185 in mouse fibroblast NIH3T3 cells (8, 9). These observations suggest that Shc proteins may play an important role in transmitting the oncogenic signal of p185 in breast cancer. The mammalian *Shc* locus encodes three overlapping isoforms of 46, 52, and 66 kDa (p46^{Shc}, p52^{Shc}, and p66^{Shc}) (7). p46^{Shc} and p52^{Shc} arise from the use of alternative translation initiation sites within the same transcript. p46^{Shc} is an amino-terminal 59-amino acid truncation of p52^{Shc}. In contrast, p66^{Shc} most likely arises from an alternatively spliced transcript, since there is only *Shc* gene and the antibodies specific to the carboxyl-terminus cross-react with all three Shc isoforms (7). Although the importance of Shc in signal transduction of receptor tyrosine kinases has been extensively investigated, the functional differences of the three isoforms remain unclear. To study the functional role of Shc in breast cancer cells that overexpress *HER2/neu,* we first compared the expression levels of Shc proteins in 10 different breast cancer cell lines that overexpress or have basal level expression of *HER2/neu.* To our surprise, we found that no or a very little amount of p66^{Shc} was detected in the six p185-overexpressing cell lines, whereas significant amounts of p66^{Shc} existed in the other four cell lines that expressed p185 at basal levels. In contrast, the levels of p46^{Shc} and p52^{Shc} expression were comparable in each of the 10 cell lines. It is interesting that the inverse correlation of expression

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of p185 and $p66^{Shc}$ appeared to be cell type specific, since $p66^{Shc}$ was detected in human ovarian, lung, and oral cancer cells and in mouse fibroblast cells irrespective of the p185 expression level. We demonstrated, furthermore, that $p66^{Shc}$ was not required for mediating the oncogenic signal of HER-2/neu to downstream signaling pathways in human breast cancer cells.

METHODS

Cell lines and culture. The human cancer cell lines used in this study were from ATCC (American Type Culture Collection, Rockville, MD). DHFR/G8 cells were derived from NIH3T3 cells expressing the transfected rat *HER2/neu* gene (3). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum under an atmosphere of 5% CO_2 at 37°C.

Antibodies. The monoclonal antibody c-neu-Ab3 targeting the carboxyl-terminal domain of p185 was obtained from Oncogene Science. The recombinant antiphosphotyrosine antibody (RC20), the monoclonal anti-Grb2 antibody, and the rabbit anti-Shc polyclonal antibody were purchased from Transduction Laboratories.

Immunoprecipitation and Western blotting. Cells were harvested at subconfluence and subjected to lysis in 1% Triton X-100, 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP-40, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Protein concentrations were determined against standardized controls using the Bio-Rad protein assay kit. Lysates were immunoprecipitated with appropriate antibodies according to the suppliers' specifications and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked with 5% dry milk powder in TPBS (0.05% Tween 20 in PBS), incubated with appropriate antibodies, incubated with goat anti-mouse or goat anti-rabbit IgG-HRP (Boehringer-Mannheim) and visualized by the ECL chemiluminescence system.

Stripping of Western blots. Used immunoblots were stripped by incubation with 62.5 mM Tris.Cl (pH 6.8), 2% SDS and 100 mM β-mercaptoethanol at 75°C for 30 min. Filters were then washed twice with TPBS and reprobed with other antibodies.

RESULTS AND DISCUSSION

To study the functional role of Shc proteins in *HER2/neu*-overexpressing breast cancer cells, we set out to evaluate the expression levels of Shc isoforms in 10 different breast cancer cell lines that express *HER2/neu* at various levels (10, 11). We first confirmed the p185 levels in these cells by immunoblot analysis with the monoclonal anti-p185 antibody. As shown in figure 1 (top panel), the p185 levels were readily detectable in the six *HER2/neu*-overexpressing cell lines, while under the same conditions the other four cell lines had no detectable p185. The p185 protein levels in these cell lines were generally consistent to previously described data on *HER2/neu* mRNA expression levels, with one exception. Previous data showed the *HER2/neu* mRNA levels in the SKBR-3 and

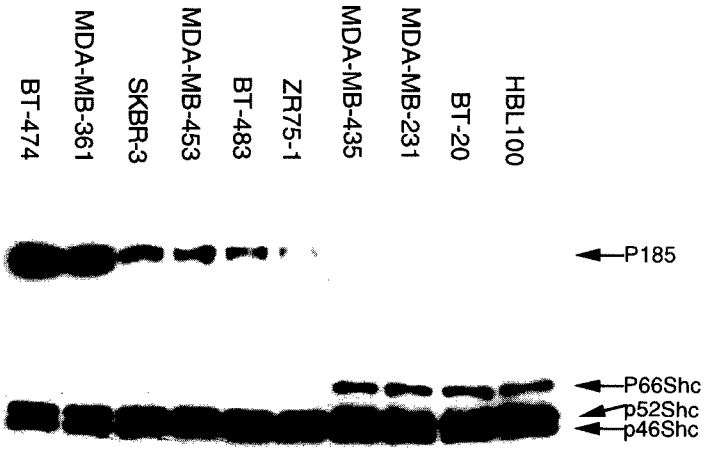

FIG. 1. Inverse correlation of expression of p185 and $p66^{Shc}$ in breast cancer cells. Fifty micrograms of cell extracts from various cell lines as indicated were subject to 6% SDS/PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-neu-mAb3, a monoclonal anti-p185 antibody, the lower part was incubated with polyclonal anti-Shc antibody.

MDA-MB-361 cell lines to be 128-fold and 64-fold over that of HBL100, respectively. Our western blot data indicated that the p185 protein level in MDA-MB-361 was higher than that in SKBR-3. This discrepancy may be due to heterogeneity in human breast cancer cells (12).

We compared the Shc protein levels in these cells with the anti-Shc polyclonal antibody (Fig. 1, bottom panel). To our surprise, the $p66^{Shc}$ isoform was clearly detected only in the four p185 basal level cell lines. No appreciable amounts of $p66^{Shc}$ were observed in any of the p185-overexpressing cell lines. Only a very faint band corresponding to the position of $p66^{Shc}$ was detected in the lanes of SKBR3 and MDA-MB-361 after longer exposure (data not shown). In contrast, the $p52^{Shc}$ and $p46^{Shc}$ protein levels were comparable in each of the 10 cell lines. These observations suggest an inverse correlation of expression of p185 and $p66^{Shc}$ in breast cancer cells.

To determine whether this inverse correlation of expression of p185 and $p66^{Shc}$ exists in other cell types, we examined by western analysis the $p66^{Shc}$ protein levels in human ovarian cancer cells and mouse fibroblast cells that overexpress *HER2/neu.* As shown in figure 2 (top panel), human ovarian cancer cell lines OVCAR3 and SKOV3 overexpress p185, while human ovarian cancer cell line 2774 does not express p185. DHFR/G8 is a NIH3T3-derived cell line overexpressing the rat *HER2/neu* gene (3). Down-regulation of $p66^{Shc}$ was not observed in cell lines OVCAR3, SKOV3, or DHFR/G8 cells (Fig. 2, bottom panel). The extra faint band on the top of $p52^{Shc}$ in some lanes was not always detectable in separate experiments. It is not yet clear whether this extra band represents an unidentified minor isoform of Shc. Since these cell lines have p185 levels higher than or similar to those of breast cancer cells tested in this study (Fig. 2 and data not shown), the appearance of $p66^{Shc}$ in these cell lines indicated that the inverse correlation of expression of p185 and $p66^{Shc}$ is not ubiquitous but cell type specific. Furthermore, we did not detect this inverse correlation in p185-overexpressing human oral cancer cell lines (13) or in stable transfectants derived from human lung cancer cells expressing *HER2/neu* cDNA (14) (data not shown). Taken together, these data suggest that the inverse correlation of p185 overexpression and $p66^{Shc}$ expression may be limited to human breast cancer cells.

To date the functional differences of the three Shc isoforms have not been investigated, probably because of an inability to clone the $p66^{Shc}$ cDNA. Our previous data have shown that the activated p185 can induce tyrosine phosphorylation of all three Shc isoforms and subsequent Shc/Grb2

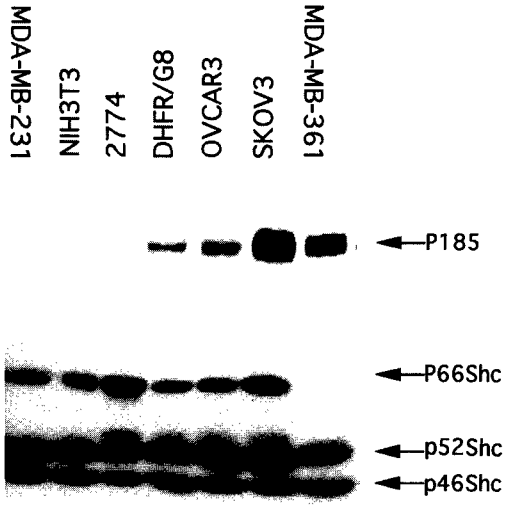

FIG. 2. Down-regulation of $p66^{Shc}$ is cell type specific. Fifty micrograms of cell lysates from different cell lines as indicated were subject to 6% SDS/PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-neu-mAb3, the lower part was incubated with polyclonal anti-Shc antibody.

complex formation and further downstream signaling pathways in fibroblast cells (8). It is also known that the p185 tyrosine kinase is activated in p185-overexpressing cells (15, 16). The absence of $p66^{Shc}$ in p185-overexpressing breast cancer cells therefore led us to examine whether the $p52^{Shc}$ and $p46^{Shc}$ isoforms can be tyrosine phosphorylated by the activated p185 in these cells and whether, in the absence of $p66^{Shc}$, they can form complex with Grb2 to mediate downstream signal pathways. We used the anti-Shc polyclonal antibody to immunoprecipitate Shc and associated proteins from two p185-overexpressing cell lines, SKBR3 and MDA-MB-453, and two p185 basal level control lines, MDA-MB-231 and MDA-MB-435. After immunoprecipitation, the immunocomplexes were separated by SDS/PAGE and subjected to western analysis with anti-p185 (Fig. 3a), recombinant anti-phosphotyrosine antibody (RC20) (Fig. 3b), or monoclonal anti-Grb2 antibody (Fig. 3c). As shown in figure 3a, Shc proteins were physically associated with the activated p185 in SKBR3 and MDA-MB-453 cells, which is similar to our previous data showing the complex formation of p185 and Shc proteins in mouse fibroblast cells expressing the point mutation-activated *HER2/neu* (8). Both $p52^{Shc}$ and $p46^{Shc}$ were tyrosine phosphorylated in SKBR3 and MDA-MB-453 cells, while no appreciable amounts of tyrosine phosphorylated Shc proteins were detected in either MDA-MB-231 or MDA-MB-435 cells. Furthermore, the Shc/Grb2 complex formed in SKBR3 and MDA-MB-453 cells but not in the two control cell lines. To show equal loading, we stripped the filter (used in panel b) and re-probed it with the anti-Shc polyclonal antibody. As shown in figure 3d, equal amounts of various cell extracts were applied in the immunoprecipitation. This experiment again demonstrated that $p66^{Shc}$ was undetectable in either SKBR3 or MDA-MB-453 cells. A recent report already showed that the downstream MAP kinase signaling pathway is activated in *HER-2/neu*-overexpressing human breast cancer cells including SKBR3 and MDA-MB-453 (17). Taken together, these observations suggest that a lack of $p66^{Shc}$

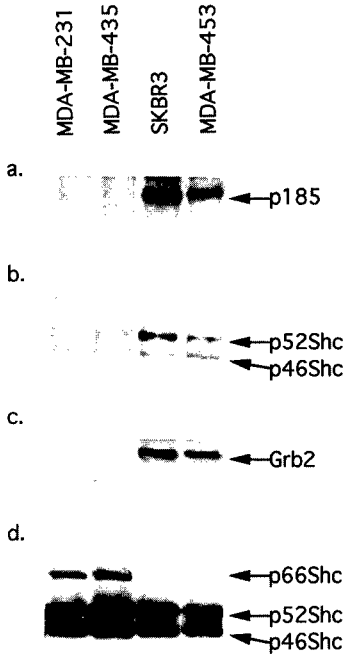

FIG. 3. Tyrosine phosphorylation of $p52^{Shc}$ and $p46^{Shc}$ by the activated p185 in breast cancer cells. Two milligrams of lysates from each cell line were immunoprecipitated with polyclonal anti-Shc antibody. Immunocomplexes were dissected by 10% SDS/PAGE, followed by immunoblotting analysis with either c-neu-Ab3 (panel a), recombinant anti-phosphotyrosine antibody (panel b), or monoclonal anti-Grb2 antibody (panel c). The filter used in panel b was stripped off and re-probed with polyclonal anti-Shc antibody (panel d). Equal loading was confirmed in panel d.

does not affect tyrosine phosphorylation of $p52^{Shc}$ and $p46^{Shc}$, Shc/Grb2 complex formation, or the downstream MAP kinase signaling pathway induction by the activated p185 in breast cancer cells. In other words, $p66^{Shc}$ is not required for mediating the oncogenic signal of p185 in human breast cancer cells that overexpress *HER-2/neu.*

It is generally believed that Shc proteins are important signal mediators for receptor tyrosine kinases. However, little is known about the distinct function of any of the three Shc isoforms. All three isoforms appear to bind and be tyrosine phosphorylated by the activated p185 and EGF receptor in fibroblast cells (7–9). A recent report demonstrated that the major insulin-dependent Shc signaling pathway occurred through the $p52^{Shc}$ isoform, whereas the EGF receptor displayed similar substrate specificity for both the $p52^{Shc}$ and the $p46^{Shc}$ forms (18). This suggests that preferential tyrosine phosphorylation of Shc isoforms may underlie physiological specificity in the signaling pathways of different receptor tyrosine kinases. Differential physiological specificity among the Shc isoforms is further supported by the fact that $p52^{Shc}$ and $p66^{Shc}$, but not $p46^{Shc}$, are associated with the PEST tyrosine phosphatase (19,20). This interaction can be enhanced by protein kinase C activator phorbol 12-myristate 13-acetate and G protein receptor agonist, but not by EGF or serum, suggesting cross-talk between the G protein receptor and the Shc signaling pathway. In addition, $p66^{Shc}$ was not expressed in any human hematopoietic cell lines tested (7), which is similar to the situation in *HER2/neu*-overexpressing breast cancer cells described in this study. It is unclear whether a common mechanism leads to the absence of $p66^{Shc}$ in hematopoietic cell lines and p185-overexpressing breast cancer cell lines, but our data suggest that both cell type specificity and overexpression of p185 may contribute to the down-regulation of $p66^{Shc}$.

We reported here a novel finding that $p66^{Shc}$ was down-regulated in p185-overexpressing breast cancer cells. This inverse correlation of p185 overexpression and $p66^{Shc}$ expression is probably specific to breast cancer cells because this phenomenon was not observed in p185-overexpressing mouse fibroblasts or human ovarian, oral, or lung cancer cells. In contrast, the $p52^{Shc}$ and $p46^{Shc}$ isoforms were expressed at similar levels in p185-overexpressing breast cancer cell lines as in those expressing basal levels of p185. Moreover, $p52^{Shc}$ and $p46^{Shc}$ without $p66^{Shc}$ appear to be sufficient to transmit the oncogenic signal of the activated p185 in breast cancer cells. Further study of the relationship between $p66^{Shc}$ expression and p185 overexpression in breast cancer cells may help us understand differential physiological functions among the Shc isoforms.

ACKNOWLEDGMENTS

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Dominant-negative Mutants of Grb2 Induced Reversal of the Transformed Phenotypes Caused by the Point Mutation-activated Rat *HER-2/Neu**

(Received for publication, July 28, 1995, and in revised form, October 3, 1995)

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To clarify the role of the Shc-Grb2-Sos trimer in the oncogenic signaling of the point mutation-activated HER-2/neu receptor tyrosine kinase (named p185), we interfered with the protein-protein interactions in the Shc·Grb2·Sos complex by introducing Grb2 mutants with deletions in either amino- (ΔN-Grb2) or carboxyl- (ΔC-Grb2) terminal SH3 domains into B104-1-1 cells derived from NIH3T3 cells expressing the point mutation-activated *HER-2/neu*. We found that the transformed phenotypes of the B104-1-1 cells were largely reversed by the ΔN-Grb2. The effect of the ΔC-Grb2 was much weaker. Biochemical analysis showed that the ΔN-Grb2 was able to associate Shc but not p185 or Sos, while the ΔC-Grb2 bound to Shc, p185, and Sos. The p185-mediated Ras activation was severely inhibited by the ΔN-Grb2 but not the ΔC-Grb2. Taken together, these data demonstrate that interruption of the interaction between Shc and the endogenous Grb2 by the ΔN-Grb2 impairs the oncogenic signaling of the activated p185, indicating that (i) the ΔN-Grb2 functions as a strong dominant-negative mutant, and (ii) Shc/Grb2/Sos pathway plays a major role in mediating the oncogenic signal of the activated p185. Unlike the ΔN-Grb2, ΔC-Grb2 appears to be a relatively weak dominant-negative mutant, probably due to its ability to largely fulfill the biological functions of the wild-type Grb2.

The *HER-2/neu* (also known as *erbB-2*) protooncogene encodes an M_r 185,000 transmembrane glycoprotein with intrinsic tyrosine kinase activity homologous to the epidermal growth factor (EGF)[1] receptor (1–6). The transforming potential of the HER-2/neu receptor tyrosine kinase (named p185) has been well documented in both clinical analysis and experimental studies (7–11). The mechanisms of aberrant activation of p185 have also been extensively investigated (12–19). A carcinogen-induced point mutation replacing a valine residue with a glutamic acid in the transmembrane domain confers transforming ability on p185 (12). Alternatively, overexpression of the wild-type p185 can also induce neoplasia transformation (13–15). Both mutation and overexpression are believed to result in enhancing formation and stabilization of receptor dimers, which allow the p185 tyrosine kinase to maintain in its active status (16–19). However, the downstream signaling pathway relaying the oncogenic signal triggered from the abnormally activated p185 is not well defined, likely due to the absence of a consensus of its ligand (20–26).

Activation of Ras is an important convergence point in the mitogenic signaling pathway of receptor tyrosine kinases (27). A key upstream pathway leading to Ras activation by receptor tyrosine kinases has recently been established, primarily as a result of studies with the receptors for EGF, platelet-derived growth factor, and insulin (28–33). The most important components of this pathway include Shc, Grb2, and Sos. Shc stands for SH2 domain-containing α2 collagen-related proteins. The Shc family consists of three isoforms (34). The $p46^{Shc}$ and $p52^{Shc}$ isoforms come from the same transcript with different translation initiation sites. The $p66^{Shc}$ species most likely arises from a distinct transcript. Tyrosine phosphorylation of Shc provides a docking site for Grb2 which was originally identified as a growth factor receptor-bound protein (35), a mammalian homolog of *Caenorhabditis elegans* Sem-5 and *Drosophila* Drk (36, 37). Grb2 is a 24-kDa adaptor protein containing an SH2 domain flanked by two SH3 domains. Through the SH3 domains, Grb2 constitutively associates with Sos (named for the *Son of Sevenless* gene), a 150-kDa guanyl-nucleotide exchange factor for Ras (38–41), by targeting the proline-rich motif at its carboxyl terminus. Upon ligand stimulation, most receptor tyrosine kinases examined to date have been able to induce tyrosine phosphorylation of Shc, which subsequently binds to the SH2 domain of Grb2. The formation of the Shc·Grb2·Sos ternary complex has been proposed to play an important role in activating Ras (28–33). Alternatively, the Grb2·Sos complex can be directly recruited to the activated EGF receptor (43). Activation of Ras leads to stimulation of downstream kinase cascades, which at least include Raf-1/MEK/MAPK and MEKK-1/JNKK/JNK pathways (44).

Unlike EGF receptor and other receptor tyrosine kinases, the mutation-activated p185 tyrosine kinase is constitutively active in the absence of exogenously added ligand. Although activation of Ras has been proposed to play an important role in the oncogenic signaling of the mutation-activated p185 (45), coupling of p185 to Ras via Shc·Grb2·Sos or Grb2·Sos or both has not been yet determined. Our recent data and that of others indicated that tyrosine phosphorylation of Shc and formation of the Shc·Grb2 complex occurred in transformed NIH3T3 cells that express the mutation-activated p185 and human breast cancer cells that overexpress p185, which suggests that the Shc/Grb2/Sos/Ras pathway may be responsible for transmit-

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[1] The abbreviations used are: EGF, epidermal growth factor; ΔN-Grb2, amino-terminal SH3 domain deletion mutant; ΔC-Grb2, carboxyl-terminal SH3 domain deletion mutant; PAGE, polyacrylamide gel electrophoresis; wt, wild-type.

ting the oncogenic signal from the activated p185 (46, 55). One way to provide more direct evidence to support this idea is to interfere with the protein-protein interactions involved in this pathway by using dominant-negative mutants and then examine whether these mutants can reverse the transformed phenotypes caused by the activated p185. Grb2 is a central component in this pathway. On the one hand, it binds tightly to Sos through its SH3 domains; on the other hand, it can bind to Shc and probably the activated p185 as well through its SH2 domain. Interestingly, recent studies suggested that the SH2 and SH3 domains of Grb2 functioned independently (47, 48). Binding of phosphopeptides to the SH2 domain of Grb2 does not appreciably affect the association of its SH3 domains with proline-rich peptides. Conversely, binding of excessive peptides derived from Sos to Grb2 does not influence the interaction between its SH2 domain with phosphopeptides. We, therefore, reasoned that deleting one of the SH3 domains of Grb2 might create dominant-negative mutants which compete with the endogenous Grb2·Sos complex for Shc or activated p185 and block the oncogenic signaling pathway of the activated p185. To test this hypothesis we transfected either an amino-terminal or an carboxyl-terminal SH3 domain deletion mutant of Grb2 into B104-1-1 cells which are transformed NIH3T3 cells expressing the mutation-activated p185. We found that the transformed phenotypes of B104-1-1 were largely reversed by the amino-terminal SH3 domain deletion mutant of Grb2 (ΔN-Grb2). The effect of the carboxyl-terminal SH3 domain deletion mutant (ΔC-Grb2) on phenotypic reversion was much weaker. Biochemical analysis data indicated that the ΔN-Grb2 functioned as a strong dominant-negative mutant, whereas the ΔC-Grb2 seemed to be a weak one. These results support the notion that the Shc/Grb2/Sos pathway plays an important role in the oncogenic signaling of the mutation-activated p185.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—B104-1-1 cells are transformed NIH3T3 cells generated by transfection with the *neu* oncogene, originally derived from a neuro/glioblastoma cell line (49). Cells were grown in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% calf serum under an atmosphere of 5% CO_2 at 37 °C, unless otherwise indicated. Cells transfected with pSV2neo in focus forming assays as indicated below were grown under the same conditions except that G418 (500 μg/ml) was added.

Plasmids—The cosmid clone cNeu-104 carries the mutation-activated rat genomic *neu* (3). ΔN-Grb2 is an amino-terminal SH3 domain deletion mutant of Grb2. ΔC-Grb2 is a carboxyl-terminal SH3 domain deletion mutant of Grb2. The cDNAs encoding the ΔN-Grb2 and ΔC-Grb2 are driven by the cytomegalovirus promoter and preceded by the hemagglutinin epitope (HA1) tag in expression vector pCGN-Bam, which contains the hygromycin-resistant gene as a selection marker (42, 57).

Establishment of Stable Transfectants of Grb2 Deletion Mutants—B104-1-1 cells were transfected by the calcium phosphate precipitation method as described previously (50) with the plasmid pCGN-Bam or the expression vectors encoding ΔC-Grb2 or ΔN-Grb2. After transfection, cells were subject to hygromycin (300 μg/ml) selection for 10–14 days. Individual colonies were expanded and characterized for expressing appropriate truncated Grb2 products. Vector, Grb2ΔC-11, and Grb2ΔN-11 are stable transfectants derived from B104-1-1 cells, expressing vector alone, ΔC-Grb2, and ΔN-Grb2, respectively.

Focus Forming Assays—Focus forming assays were performed as described previously with some modifications (51). cNeu-104 (1 μg) was cotransfected into NIH3T3 cells with pSV2neo (0.1 μg) and 10 μg of plasmids encoding vector alone, ΔC-Grb2, or ΔN-Grb2. The filler plasmid, pGEM, was used to ensure that equal amounts of DNA were transfected into cells. Two days after transfection, cells were split 1:4, and duplicate plates were cultured in regular medium for 3–4 weeks while the other set of duplicates were grown in medium containing G418. Foci and G418-resistant colonies were stained in crystal violet solution (1% crystal violet, 20% ethanol in H_2O). The resulting number of foci from each transfection was corrected for transfection efficiency by dividing by the number of G418-resistant colonies created by the same transfection. Results are expressed as percentage of foci in control transfection with cNeu-104 (100%). Shown here is the average of three individual experiments. Standard deviation is shown by an error bar.

Microfocus Forming Assays—Microfocus forming assays were performed as described previously (52) with some modifications. Exponentially growing NIH3T3, B104-1-1, Vector, Grb2ΔC-11, and Grb2ΔN-11 cells were trypsinized and counted. One hundred fifty cells from each cell line were combined separately with 3.0×10^5 NIH3T3 cells and gently mixed in a 6-cm tissue culture plate containing regular medium. Medium was replaced every 3 days over the 2–3-week period of focus formation. Foci were counted as described above. The diameters of individual foci in a random sampling from each plate were also measured.

Colony Formation in Soft Agar—Experiments were carried as previously reported with some modifications (53). Cells (1,000/well) were plated in a 24-well plate in regular medium containing 0.35% Seaplaque-agarose on an underlay of 0.7% agarose. After being cultured for 4–6 weeks, the colonies were stained with *p*-iodonitrotetrazolium violet (1 mg/ml) and counted.

Antibodies—The monoclonal antibody c-neu-Ab3 targeting at the carboxyl-terminal domain of p185 and the monoclonal anti-Ras antibody (Y13–259) were from Oncogene Science. The monoclonal anti-phosphotyrosine antibody (PY20) was from UBI. The rabbit anti-Shc and anti-Sos polyclonal antibodies and the monoclonal anti-Grb2 antibody were purchased from Transduction Laboratories. The monoclonal anti-HA1 epitope antibody (12CA5) was from Boehringer Mannheim.

Immunoprecipitation and Western Blotting—Unless otherwise indicated, all cells were harvested at subconfluence and lysed in 1% Triton X-100, 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Protein concentrations were determined against standardized controls using the Bio-Rad protein assay kit. Lysates were immunoprecipitated with appropriate antibodies according to the suppliers' specifications, and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters (Schleicher & Schuell). Filters were blocked with 5% dry milk powder in TPBS (0.05% Tween 20 in phosphate-buffered saline), incubated with appropriate antibodies, incubated with goat anti-mouse or goat anti-rabbit IgG Fc-horseradish peroxidase (Boehringer Mannheim), and then visualized by chemiluminescence system (ECL).

Stripping of Western Blots—Used immunoblots were stripped by incubation with 62.5 mM Tris·Cl, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 75 °C for 30 min. Filters were then washed twice with TPBS and reprobed with other antibodies.

Analysis of Ras-bound Guanine Nucleotides—The analysis was performed as described in the previous reports (54), with some modifications. 2.5×10^4 cells percentimeter squared were starved overnight in phosphate-free medium supplemented with 5% dialyzed serum. Cells were then labeled for 12 h with [^{32}P]orthophosphate (400 μCi/ml) in the same medium. Cells were put on ice, rapidly washed with ice-cold phosphate-buffered saline, and lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 20 mM $MgCl_2$, 0.5% Nonidet P-40, 0.2 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Nuclei and cell debris were removed by centrifugation. Ras proteins were immunoprecipitated with Y13-259, a monoclonal antibody to Ras. A goat anti-rat secondary antibody, and protein A-agarose were also applied in the immunoprecipitation. The beads were extensively washed with lysis buffer and proteins were solubilized in 1% SDS at 68 °C. The bound guanine nucleotides were chromatographed on polyethyleneimine-cellulose plates in 1.3 M LiCl. The GTP/GDP ratio was determined by a Betascope 603 Blot Analyzer (Betagen, Boston, MA). The GTP-Ras percentage in the B104-1-1 cells was arbitrarily set at 100%. The percent GTP-Ras in other cell lines were standardized against that of the B104-1-1.

RESULTS

Suppression of the Transforming Ability of the Mutation-activated HER-2/neu by SH3 Domain Deletion Mutants of Grb2—To observe whether the SH3 domain deletion mutants of Grb2 (Fig. 1*A*) can interfere with the oncogenic signaling pathway of the mutation-activated *HER-2/neu* receptor tyrosine kinase, we first examined the effect of the Grb2 mutants on the transforming activity of the *HER-2/neu* oncogene by focus forming assays in which we cotransfected cNeu-104 (a cosmid clone containing mutation-activated rat *HER2/neu,* see

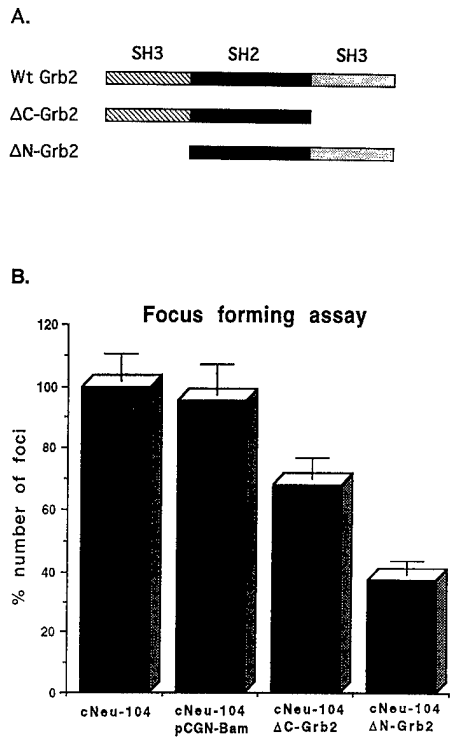

FIG. 1. *A*, schematic representation of Grb2 deletion mutants. ΔN-Grb2 represents the Grb2 mutant containing only the SH2 and the carboxyl-terminal SH3 domains. ΔC-Grb2 stands for the Grb2 mutant carrying only the SH2 and the amino-terminal SH3 domains. *B*, SH3 domain deletion Grb2 mutants suppress the transforming ability of the mutation-activated *neu*. Focus forming assays were performed as described under "Experimental Procedures." The resulting number of foci from each transfection was corrected for transfection efficiency by dividing by the number of G418-resistant colonies created by the same transfection. Results are expressed as percent of foci in control transfection with cNeu-104 (100%). Data shown here are the average from three individual experiments. Standard deviations are shown by *error bars*.

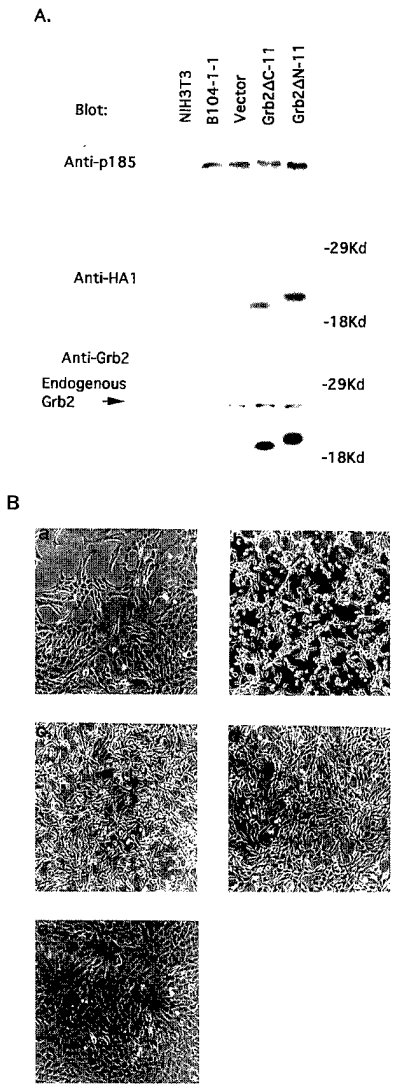

FIG. 2. **Morphologic reversion of the transformed B104-1-1 cells by ΔN-Grb2.** *A*, immunoblot analysis of expression of the Grb2 deletion mutants stably transfected into B104-1-1 cells. B104-1-1 is a transformed cell line derived from NIH3T3 by stable transfection of the point mutation-activated *HER-2/neu*. Vector, Grb2ΔC-11, and Grb2ΔN-11 are stable transfectants derived from B104-1-1 cells, expressing vector alone, ΔC-Grb2, and ΔN-Grb2, respectively. Fifty micrograms of cell extracts from various cell lines as indicated were subject to 10% SDS-PAGE. After transfer, the top portion of the nitrocellulose filter was probed with c-Neu-Ab3, a monoclonal anti-p185 antibody, the lower part was incubated with either 12CA5, a monoclonal antibody specific to the HA1 tag (*middle panel*), or monoclonal anti-Grb2 antibody (*bottom panel*). Endogenous Grb2 is indicated by an *arrowhead*. *B*, morphology of various cell lines. *a*, NIH3T3; *b*, B104-1-1; *c*, Vector; *d*, Grb2ΔC-11; and *e*, Grb2ΔN-11.

Ref. 3) together with expression vectors encoding either the amino-terminal SH3 domain deletion Grb2 (ΔN-Grb2) or the carboxyl-terminal SH3 domain deletion Grb2 (ΔC-Grb2) into NIH3T3 cells. When the expression vector encoding the ΔN-Grb2 was cotransfected with cNeu-104, the number of foci caused by cNeu-104 decreased by more than 60%. Cotransfection of the expression vector for the ΔC-Grb2 could also decrease the number of foci but only by approximately 25%. In control experiments cotransfection of cNeu-104 plus vector backbone alone did not affect the number of foci caused by cNeu-104 (Fig. 1*B*). Transfection of either expression vectors encoding the ΔN-Grb2, ΔC-Grb2, or the vector backbone alone into NIH3T3 cells did not result in any foci (data not shown). The results of focus forming assays indicated that the SH3 domain deletion mutants of Grb2 were able to suppress the transforming ability of the mutation-activated *HER-2/neu* and that ΔN-Grb2 was more effective than ΔC-Grb2.

Phenotypic Reversion of the Transformed B104-1-1 Cells by Stable Transfection of Grb2 Mutants with SH3 Domain Deletion—We next wanted to know whether expression of ΔN-Grb2 or ΔC-Grb2 could induce phenotypic reversion of established transformed cells caused by the mutation-activated *HER-2/neu*. To do so, we stably transfected expression vectors encoding ΔN-Grb2, ΔC-Grb2, or vector backbone into B104-1-1 cells, which are transformed NIH3T3 cells expressing the mutation-activated rat *HER-2/neu*. After 10–14 days of hygromycin selection, individual colonies were expanded and characterized for expressing appropriate truncated Grb2 products by Western analysis with the monoclonal antibody against the HA1 tag which was fused to the Grb2 mutants. The truncated Grb2 products are expected to be about 22 kDa. We focused our attention on two clones that showed comparable expression levels of ΔN-Grb2 and ΔC-Grb2, respectively. As shown in Fig. 2*A*, Grb2ΔN-11 is the stable transfectant expressing ΔN-Grb2, whereas Grb2ΔC-11 expresses the ΔC-Grb2. The expression levels of ΔN-Grb2 and ΔC-Grb2 are comparable between these two lines based on the Western blotting using anti-HA1 antibody. The mobility of the ΔN-Grb2 product is a little slower than the ΔC-Grb2 protein. Morphology studies indicated that the transfectant with the vector backbone alone looked similar to the transformed parental B104-1-1 cell line (Fig. 2*B*). In contrast, Grb2ΔN-11 showed morphologic reversal. This was

TABLE I
Comparison of the transformed properties of parent B104-1-1 cells and transfectants of SH3 domain deletion Grb2 mutants by microfocus forming assay and soft agar assay

Cell lines	Number of foci[a]	Size of foci[a]	Soft agar colonies[b]
	%	*mm*	
NIH3T3	0		0
B104-1-1	100	2.5–5.0	100
Vector	92.6 ± 8.5	2.5–5.0	95.1 ± 9.6
Grb2ΔC-11	70.2 ± 7.4	2.0–4.0	74.5 ± 5.8
Grb2ΔN-11	23.7 ± 2.8	1.0–2.0	20.1 ± 2.9

[a] One hundred fifty cells from each line to be tested were mixed with 3.0×10^5 NIH3T3 cells in a 6-cm tissue culture plate and grown as described under "Experimental Procedures." The number of foci was counted after 2–3 weeks. The sizes of foci were also measured. The resulting number of foci from various cell lines in each experiment was standardized against that of the parental B104–1-1 cells (set at 100%). Data shown here are the average of three separate experiments. Standard deviation is also indicated.

[b] Cells were seeded at 1×10^3 in 0.35% agarose containing Dulbecco's minimal essential medium/Ham's F-12 with 10% calf serum. Colonies were counted 4–6 weeks later. The resulting number of colonies from various cell lines was standardized against that of the parental B104–1-1 cells (set at 100%). Data shown here represent the average of quadruplicate experiments. Standard errors are also shown.

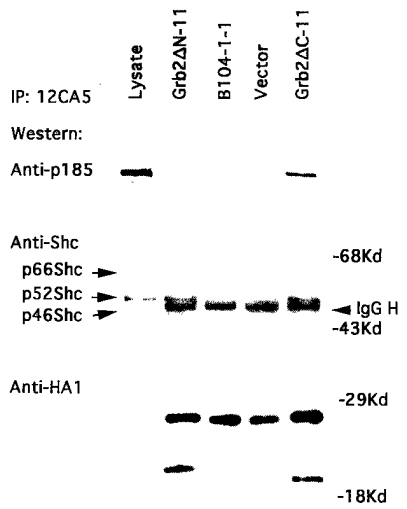

FIG. 3. **Association of Shc or p185 with the truncated Grb2 products.** Two milligrams of cell extracts from different cell lines were used in immunoprecipitation with monoclonal anti-HA1 antibody. Immunocomplexes or 50 μg of lysates from B104-1-1 cells were separated on a 6–12% gradient SDS-PAGE. The filter was cut into three pieces after transfer. The top portion was probed with c-Neu-Ab3 for associated p185, the middle was incubated with polyclonal anti-Shc antibody for associated Shc, and the bottom portion was incubated with monoclonal anti-HA1 antibody, in order to evaluate equal loading. The band above the truncated Grb2 products was most likely from IgG light chain.

evident by loss of the spindle-shaped morphology and the bright, refractile cell borders that characterize B104-1-1 cells. Moreover, Grb2ΔN-11 cells displayed an ordered growth pattern and contact inhibition, which are properties of nontransformed cells like NIH3T3 cells. The Grb2ΔC-11 cells also exhibited morphology change when compared to the B104-1-1 cells (Fig. 2*B*). But the morphology change did not reflect a complete reversion like that of the Grb2ΔN-11 cells.

To ascertain whether *HER-2/neu* was still expressed in the stable transfectants, we performed immunoblot analysis with c-neu-Ab3, a monoclonal antibody specific to p185. As shown in Fig. 2*A*, the expression level of p185 was comparable between the parental B104-1-1 and its transfectants, indicating that the morphologic reversion seen in Grb2ΔN-11 cells was not due to spontaneous loss of the *HER-2/neu* gene or down-regulation of *HER-2/neu* expression by the ΔN-Grb2 mutant. Similarly, the levels of endogenous Grb2 were comparable between the parental B104-1-1 line and its transfectants along with NIH3T3 cells as shown in Western analysis with anti-Grb2 monoclonal antibody (Fig. 2*A*). Both ΔC-Grb2 and ΔN-Grb2 were also recognized by the same anti-Grb2 monoclonal antibody (Fig. 2*A*).

To evaluate more precisely the phenotypic reversion caused by the SH3 domain deletion Grb2 mutants, we performed microfocus forming assays (52) and soft agar colony formation assays to compare the transformed properties of the parental B104-1-1 cell line and its stable transfectants. As shown in Table I, the focus formation efficiency of Grb2ΔN-11 cells dramatically decreased. For example, the number of foci formed by the Grb2ΔN-11 cells was less than 25% that of the parental B104-1-1 cells. In addition, the size of foci formed by the Grb2ΔN-11 cells were much smaller than the parental B104-1-1 cells. As expected, the Vector control cell line displayed a formation efficiency similar to the B104-1-1 cell line. The Grb2ΔC-11 cell line showed only a 30% reduction in focus formation when compared to the parental B104-1-1 line and the foci were slightly smaller. Consistent with the data from the microfocus forming assays, soft agar colony formation assays also indicated that the transformation potency of the Grb2ΔN-11 cells was significantly weakened, while the Grb2ΔC-11 cells were only moderately affected (Table I). The phenotypic reversion observed in the microfocus forming assays and soft agar colony assays was not due to the decreased growth rates of Grb2ΔN-11 and Grb2ΔC-11 cells demonstrated in *in vitro* growth rate assays (data not shown), since extending the culture time for these two cell lines in the above assays did not result in extra numbers of foci or colonies (data not shown). Taken together, our data indicated that the transformed phenotypes of B104-1-1 cells could be largely reversed by stable transfection of the ΔN-Grb2 mutant. The ΔC-Grb2 mutant had a relatively weak effect.

Both ΔN-Grb2 and ΔC-Grb2 Compete with the Endogenous Grb2 for Shc—To investigate the molecular basis of the phenotypic reversion caused by the SH3 domain deletion Grb2 mutants, we first compared the tyrosine phosphorylation profiles between B104-1-1 and its stable transfectants. Immunoblotting analysis indicate that no obvious difference for the profiles of tyrosine phosphorylation was detected between B104-1-1 and Vector, Grb2ΔN-11, or Grb2ΔC-11 (data not shown). In particular, expression of ΔN-Grb2 or ΔC-Grb2 did not affect the expression and tyrosine phosphorylation of p185 and Shc (data not shown). We next tested the possibility that the SH3 domain deletion Grb2 mutants may compete with the endogenous Grb2 for Shc and p185. We first examined whether ΔN-Grb2 and ΔC-Grb2 could be associated with Shc and p185 by co-immunoprecipitation Western analysis. Anti-HA1 antibody was used to precipitate the ΔN-Grb2 and ΔC-Grb2 proteins along with their associated proteins. As shown in Fig. 3, comparable amounts of $p52^{Shc}$ were detected in the anti-HA1 immunoprecipitates in both Grb2ΔN-11 and Grb2ΔC-11 cells. Immunoblot analysis with anti-HA1 antibody indicated that comparable amounts of ΔN-Grb2 and ΔC-Grb2 were precipitated, which suggests that the ΔN-Grb2 and the ΔC-Grb2 have a similar affinity for Shc. In contrast, co-precipitation of p185 by the anti-HA1 antibody was only detected in the Grb2ΔC-11 cells. The reciprocal co-immunoprecipitation experiment with anti-p185 antibody also failed to detect a physical association between p185 and the ΔN-Grb2 in the Grb2ΔN-11 cells (data not shown). These results suggest that the ΔN-Grb2 may not be able to associate with p185. However, we cannot rule out the possibility that the association is transient and undetectable under our experimental conditions.

Detecting the association between Shc and the truncated

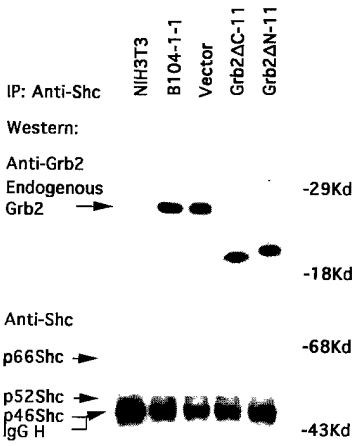

FIG. 4. **The association between Shc and the endogenous Grb2 is impaired by both ΔN-Grb2 and ΔC-Grb2.** Two milligrams of lysates from each cell line were immunoprecipitated with a polyclonal anti-Shc antibody. Immunocomplexes were dissected by 10% SDS-PAGE, followed by immunoblotting analysis with either monoclonal anti-Grb2 antibody (*top panel*) or polyclonal anti-Shc antibody (*lower panel*). Immunoprecipitated Shc proteins and co-immunoprecipitated endogenous Grb2 are indicated.

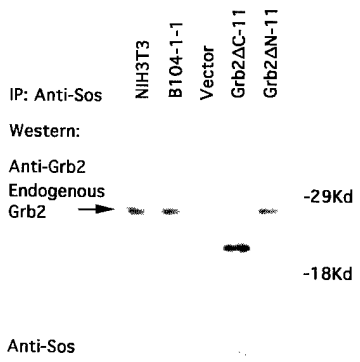

FIG. 5. **Endogenous Grb2·Sos association is interfered with the ΔC-Grb2 but not the ΔN-Grb2.** One milligram of lysate from each cell line as indicated was incubated with polyclonal anti-Sos antibody. Immunocomplexes were separated by 10% SDS-PAGE, followed by Western analysis with either monoclonal anti-Grb2 (*upper panel*) or polyclonal anti-Sos antibody (*lower panel*).

Grb2 proteins prompted us to ask whether the interaction of Shc and the endogenous Grb2 is inhibited in Grb2ΔN-11 and Grb2ΔC-11 cells. To address this issue, we used anti-Shc antibody to precipitate Shc and associated proteins, followed by immunoblotting with anti-Grb2 or anti-Shc antibodies. As shown in Fig. 4, the endogenous Grb2 co-precipitated by the anti-Shc antibody dramatically decreased in both Grb2ΔN-11 and Grb2ΔC-11 cell lines, as compared to that in the parental B104-1-1 and the vector control cell lines. Consistently, co-immunoprecipitation of the ΔC-Grb2 and ΔN-Grb2 by anti-Shc antibody was detected by the anti-Grb2 antibody. Equal loading was confirmed by Western analysis with the anti-Shc antibody. These results are consistent with that seen in Fig. 3, which showed that both ΔN-Grb2 and ΔC-Grb2 products bound to $p52^{Shc}$. We, therefore, concluded that both ΔN-Grb2 and ΔC-Grb2 were able to compete with the endogenous Grb2 for Shc.

The Association between Sos and Endogenous Grb2 Was Impaired by the ΔC-Grb2 but Not the ΔN-Grb2—Grb2 has been shown to bind constitutively to Sos even in the absence of extracellular stimuli (38). We wondered whether the Grb2/Sos association could be interfered with by ΔN-Grb2 or ΔC-Grb2. To answer this question we used anti-Sos antibody to co-precipitate endogenous Grb2 from NIH3T3, B104-1-1, Vector, Grb2ΔN-11, and Grb2ΔC-11 cells. As expected, the association of Grb2 and Sos was comparable in NIH3T3, B104-1-1, and Vector cells (Fig. 5). In contrast, the association of Sos and the endogenous Grb2 was inhibited in the Grb2ΔC-11 cells. This interference was most likely due to the competition for Sos between the endogenous Grb2 and the ΔC-Grb2 since the anti-Sos antibody was able to co-precipitate ΔC-Grb2. In contrast from the ΔC-Grb2, the ΔN-Grb2 did not significantly affect the association of Sos and the endogenous Grb2 in the Grb2ΔN-11 cells (Fig. 5). Converse immunoprecipitation with the anti-HA1 antibody also failed to co-precipitate appreciable amounts of Sos in the Grb2ΔN-11 cells (data not shown), suggesting that the association of ΔN-Grb2 with Sos is very weak. Our results are consistent with a previous report which demonstrated that substitution of Gly-203 with Arg in the carboxyl-terminal SH3 domain of Grb2 had little effect on its binding to Sos *in vitro*, whereas replacement of Pro-49 with Leu in the amino-SH3 domain (a mutation causing loss of function in *C. elegans* Sem-5) abrogated this binding (39). However, some previous reports claimed that binding of Grb2 to Sos depended on cooperative actions of the two Grb2 SH3 domains (38, 41). This discrepancy may be an issue of binding affinity.

Effect of Grb2 Mutants on Ras Activation—It has been proposed that formation of the Shc·Grb2·Sos ternary complex plays an important role in Ras activation triggered by activation of the EGF receptor, platelet-derived growth factor receptor, or insulin receptor (28–33). Given that both ΔC-Grb2 and ΔN-Grb2 mutants can compete with the endogenous Grb2 for Shc and that ΔC-Grb2 can also interact with p185 and Sos (Figs. 3–5), we examined whether signaling from the activated p185 to Ras was affected in Grb2ΔN-11 and Grb2ΔC-11 cells. Cells were labeled with $^{32}P_i$, and the guanine nucleotides bound to Ras were analyzed. As expected, the Vector control cells had a similar percentage of GTP-Ras, as compared to the B104-1-1 cells (Fig. 6). In contrast, the percentage of GTP-Ras in the Grb2ΔN-11 cells was less than 35% of that in the parental B104-1-1 cells while the percentage of GTP-Ras decreased to about 75% in the Grb2ΔC-11 cells. These results indicate that interruption of the interaction between Shc and the endogenous Grb2 can inhibit Ras activation by the activated p185 and that the ΔN-Grb2, which does not bind to p185 or Sos, acts as a strong dominant-negative analog of the endogenous Grb2. The ΔC-Grb2 mutant appears to be a relatively weak dominant-negative mutant, probably because it is still able to form complexes with Sos, Shc, and p185. The differential inhibition effect of ΔN-Grb2 and ΔC-Grb2 on Ras activation is consistent to the different extent of phenotypic reversion of B104-1-1 cells caused by these two Grb2 mutants. These observations support the idea that Ras activation is a key event in the process of transformation induced by the mutation-activated p185.

DISCUSSION

Grb2 consists of a single SH2 domain and two SH3 domains. Previous studies have indicated that Grb2 is a key component of the pathway leading to Ras activation by receptor tyrosine kinases (56). In the present study we tested whether deletion of the amino- or carboxyl-SH3 domain of Grb2 could create dominant-negative mutants which are capable of binding to tyrosine-phosphorylated Shc or mutation-activated p185 but are unable to associate with Sos. We speculated that these mutants could interfere with the recruitment of the Sos·Grb2 complex to Shc or p185, leading to inhibition of Ras activation. Our data demonstrated here that the ΔN-Grb2 functioned as a dominant-negative mutant that suppressed by more than 65% the activation of Ras by the mutation-activated p185 and largely reversed the transformed phenotypes of B104-1-1 cells. The

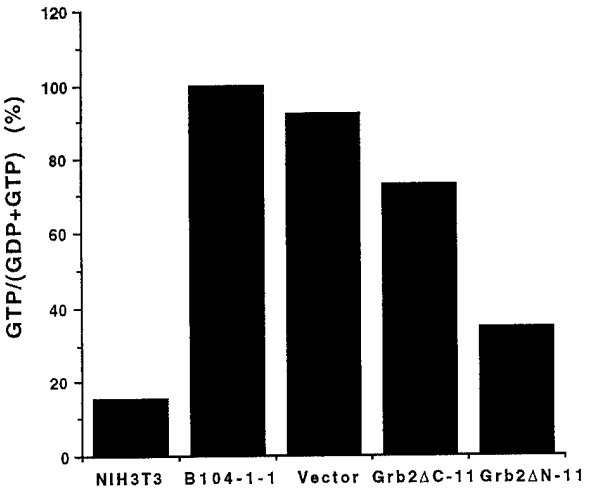

FIG. 6. **Effect of expression of SH3 domain deletion mutants of Grb2 on Ras activation.** Cells were labeled with $^{32}P_i$ in phosphate-free medium at 25,000 cells/cm^2. Cell lysates were prepared and subjected to immunoprecipitation with anti-Ras monoclonal antibody Y13-259. Guanine nucleotides precipitated with Ras were separated with thin-layer chromatography. The amount of Ras-GTP was expressed as a percentage of the amount of Ras-GDP plus Ras-GTP. The GTP-Ras percentage in the B104-1-1 cells was arbitrarily set at 100%. The percentage of Ras-GTP in other cell lines were standardized against that of the B104-1-1. Data shown here represent the average of two separate experiments. Deviations are less than 10%.

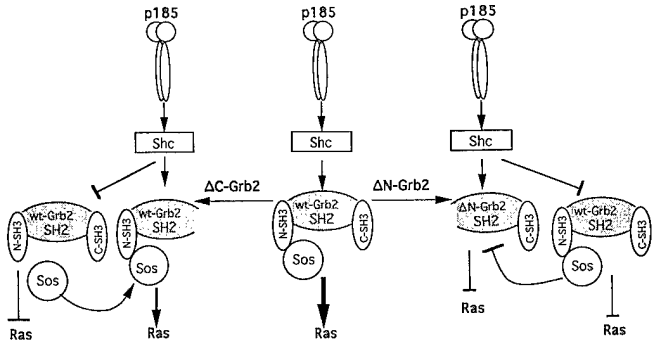

FIG. 7. **Hypothetic working modes of wild-type Grb2 and its SH3 domain deletion mutants.** Wild-type Grb2 (wt-Grb2) constitutively binds to Sos mainly through its amino-terminal SH3 domain. The Grb2·Sos complex is recruited to the Shc that is tyrosine phosphorylated, leading to Ras activation. After introduced into the B104-1-1 cells, the ΔN-Grb2 can bind to Shc but not Sos. The Shc·ΔN-Grb2 complex by itself is unable to trigger Ras activation. On the other hand, ΔN-Grb2 sequesters Shc. Therefore, the endogenous wt-Grb2 cannot be recruited to Shc. Thus, ΔN-Grb2 is a dominant-negative mutant of Grb2. In contrast, the ΔC-Grb2 binds to Sos and Shc. The Shc·ΔC-Grb2·Sos complex can largely fulfill the functions of the Shc·wt-Grb2·Sos complex, leading to Ras activation. Since the recruitment efficiency of ΔC-Grb2·Sos by Shc is relatively lower as compared to that of the wt-Grb2·Sos, Ras activation is slightly reduced in B104-1-1 transfectant expressing the ΔC-Grb2. In order to simplify the model, direct recruitment of wt-Grb2 or ΔC-Grb2 to the mutation-activated p185 is not included in this model, which is described in the text instead.

ΔC-Grb2 appears to be a weak dominant-negative mutant. It down-regulated Ras activation, by only 25%, and slightly induced phenotypic reversion of the B104-1-1 cells. Similar results have been recently obtained for the oncogenic Bcr-Abl tyrosine kinase (57). A ΔN-Grb2 mutant suppresses Bcr-Abl-mediated Ras activation and reverses the transformed phenotype. As shown here, the ΔC-Grb2 mutant is less effective in reversing Bcr-Abl-induced transformation as compared to the ΔN-Grb2.

It is of interest to note that the dominant-negative effect of the ΔC-Grb2 is much weaker than that of the ΔN-Grb2 even though the ΔC-Grb2 is able to compete with the endogenous Grb2 for Shc, p185, and Sos while the ΔN-Grb2 can only bind to Shc. One possible model to explain this phenomenon is shown in Fig. 7. Wild-type Grb2 (wt-Grb2) constitutively binds to Sos mainly through its amino-terminal SH3 domain. The wt-Grb2·Sos complex is recruited to the tyrosine-phosphorylated Shc, subsequently resulting in Ras activation. The wt-Grb2·Sos may also be directly recruited to the activated p185, which is not shown here in order to simplify the model (discussion seen in the text). When introduced into the B104-1-1 cells, the ΔN-Grb2 sequesters tyrosine-phosphorylated Shc. Therefore, recruitment of the wt-Grb2·Sos complex to Shc is severely impaired. Furthermore, ΔN-Grb2 cannot appreciably bind to Sos. Thus the complex of Shc and ΔN-Grb2 cannot lead to Ras activation. Taken together, the ΔN-Grb2 inhibits Ras activation mediated by the Shc/wt-Grb2/Sos pathway. Unlike the ΔN-Grb2, the ΔC-Grb2 can bind to both tyrosine-phosphorylated Shc and Sos. Deletion of the carboxyl-terminal SH3 domain of Grb2 may not significantly affect its biological functions. In other words, the exogenously expressed ΔC-Grb2 by itself may largely fulfill the functions of the wt-Grb2. Therefore, Ras activation is not dramatically shut down and the transformed phenotypes of the B104-1-1 cells are not obviously reversed even though the endogenous Grb2 is largely competed out from the Shc/Grb2/Sos pathway by the ΔC-Grb2. This model is indirectly supported by previous studies on the *C. elegans* Sem-5. The mutation in Sem-5 (*sem-5* allele n2195) corresponding to the G203R Grb2 mutant (C-terminal SH3) had a much weaker phenotypic effect in *C. elegans* than the mutation (*sem-5* allele n1619) corresponding to the P49L Grb2 mutant (N-terminal SH3) (59). However, other studies suggest that both P49L and G203R Grb2 were loss-of-function mutants (35). For example, co-microinjection of either P49L or G203R Grb2 protein together with the H-ras protein did not stimulate DNA synthesis in quiescent rat embryo fibroblast cells while coinjection of the wild-type Grb2 and H-ras proteins enhanced DNA synthesis, suggesting that the two SH3 domains of Grb2 constitute an essential functional component of the protein. These conflicting observations may be explained by the use of different assays in different biological systems. The functional difference between ΔC-Grb2 and G203R Grb2 is not known currently. We speculate that the ΔC-Grb2 possesses, at least in part, the biological functions of the wild-type Grb2 since it can bind to Shc, p185, and Sos. The slight down-regulation of Ras activation and partial reversal of transformed phenotypes in Grb2ΔC-11 cells may be due to the relatively lower efficiency of recruitment of the Sos·ΔC-Grb2 complex to Shc, as compared to the complex of Sos and the endogenous Grb2. This is supported by the observation seen in Fig. 4 in which comparable amounts of the ΔC-Grb2 in the Grb2ΔC-11 cells and the endogenous Grb2 in the B104-1-1 cells were co-precipitated by the anti-Shc antibody even though the expression level of the ΔC-Grb2 was much higher in the Grb2ΔC-11 cells than that of the endogenous Grb2 in B104-1-1 cells (Fig. 2*A*). This finding suggests that the ΔC-Grb2 may have a lower affinity for Shc than does the endogenous Grb2. Alternatively, the nucleotide exchange activity of Sos may be relatively weaker in the Sos·ΔC-Grb2 complex than in the Sos-endogenous Grb2 complex, probably because of an unknown allosteric effect. Therefore, Ras activation in the Grb2ΔC-11 cells is not as efficient as that in the B104-1-1 cells, leading to slight reversal of the transformed phenotypes caused by the mutation-activated p185.

Previous studies on the EGF receptor signaling pathway indicated that the Grb2·Sos complex could be recruited to tyrosine-phosphorylated Shc or directly to the activated EGF receptor (38–41). The data presented here imply that the Shc/Grb2/Sos pathway is most likely the dominant one coupling the

activated p185 to Ras since interference of the interaction between Shc and Grb2 by ΔN-Grb2 leads to a dramatic inhibition of Ras activation. This idea is consistent with our previous observation that deletion of most of the autophosphorylation sites, including the potential Grb2 binding site on the mutation-activated p185 did not affect its transforming ability, suggesting that direct binding of Grb2 to p185 is not essential for Ras activation (46). Similarly, recent studies using peptide competition and immunodepletion approaches also demonstrated that formation of a complex of EGF receptor with Grb2 was only responsible for a minor part of EGF-stimulated Ras activation while the formation of the Shc·Grb2·Sos complex played the major role (28, 29). Indirect evidence has been shown suggesting that Grb2 binding to tyrosine-phosphorylated Shc is more important than Grb2 binding to the insulin receptor substrate-1 in the activation of Ras in response to insulin (30, 31).

Our data, however, also suggest that the Shc/Grb2/Sos pathway may not be the sole pathway that leads to the activation of Ras by the mutation-activated p185. Disruption of the association between Shc and Grb2 by ΔN-Grb2 is unable to completely inhibit Ras activation or to completely reverse the transformed phenotypes mediated by the mutation-activated p185, suggesting the existence of multiple routes to Ras, which may not be influenced by the ΔN-Grb2. One conceivable pathway is the direct recruitment of Grb2·Sos to the activated p185 since ΔN-Grb2 appears to be unable to compete with the endogenous Grb2 for p185. Alternatively, the formation of complexes containing Grb2 and phosphorylated proteins other than Shc, which can stimulate the Ras pathway, may not be interfered with by the ΔN-Grb2. It has been shown that a complex of Syp/SH-PTP2 tyrosine phosphatase and Grb2 can couple platelet-derived growth factor receptors to Ras (60). Recently, a Ras-GAP associated protein, named p62, has been found to form a complex with Grb2 in v-*src* transformed NIH3T3 cells (61). Interestingly, the presence of the Grb2·p62 complex correlates with the phosphorylation of p62 and cellular transformation, suggesting that the Grb2·p62 complex may be able to lead to Ras activation. It will be of interest to test whether these complexes exist in the B104-1-1 cells and whether ΔN-Grb2 and ΔC-Grb2 are able to interfere with these pathways. Furthermore, the existence of other potential pathways to Ras, in which Grb2 or Sos is not involved, may also account for the absence of complete Ras inhibition and incomplete phenotypic reversion induced by the ΔN-Grb2. It is now known that mammalian cells contain several Ras guanine nucleotide exchange factors (GEF) apart from Sos. One of these, C3G (named for *C*rk *SH3* binding *GEF*), can activate Ras in yeast (62). Intriguingly, via its proline-rich domain C3G binds the amino-terminal domain of the adaptor protein Crk (62, 63). The Crk·C3G complex may thus, like the Grb2·Sos complex, couple the oncogenic signal of the mutation-activated p185 to Ras. However, no data exist showing that C3G is an exchange factor for Ras in mammalian cells. On the other hand, an alternative explanation for the failure to completely reverse the transformed phenotypes of B104-1-1 cells by the ΔN-Grb2 could be that additional, perhaps less efficient, signaling pathways which do not involve Ras are not influenced by the ΔN-Grb2 and may culminate in cell transformation by the activated p185. Indeed, recent studies have shown that Raf can be activated by the *Drosophila torso* receptor tyrosine kinase in a Ras-independent pathway (58). Our studies provided direct evidence to support the hypothesis that the Shc/Grb2/Sos pathway plays a major role in the oncogenic signaling of the mutation-activated p185 and may shed light on developing therapeutic agents to block the oncogenic signaling pathway of the p185 oncoprotein.

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Mutant SV40 large T antigen as a therapeutic agent for HER-2/*neu*–overexpressing ovarian cancer

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The HER-2/*neu* gene is frequently amplified and/or its protein product, p185, is overexpressed in a number of human cancers. Overexpression of p185 correlates with poor prognosis and low survival rates in ovarian cancer patients. We previously found that the K1 mutant of SV40 large T antigen inhibits rat *neu* promoter and suppresses mutation-activated rat *neu* transformation in mouse fibroblasts. We show here that K1 also inhibits human HER-2/*neu* promoter in human ovarian cancer cells. To investigate whether K1 can suppress HER-2/*neu* transformation and thus is a potential therapeutic agent, we used an orthotopic ovarian cancer model in which mice were injected intraperitoneally with HER-2/*neu*–overexpressing human ovarian cancer cells to induce tumor development. The tumor-bearing mice were then treated with K1-liposome complex weekly. We found that liposome-mediated K1 gene transfer decreased the p185 protein level by K1 expression in these cancer cells and significantly prolonged mice survival; about 40% of these treated mice were alive for more than 1 year without any tumor development. On the other hand, the animals from control groups that did not receive this gene therapy all developed tumors and died within 7 months. The results indicate that liposome-mediated K1 gene transfer is able to suppress tumor development from HER-2/*neu*–overexpressing ovarian cancer cells in mice.

Key words: HER-2/neu; mutant SV40 large T; ovarian cancer; gene therapy; liposome.

Ovarian cancer is one of the major malignant diseases in women. It has a high rate of mortality because of the difficulties in early diagnosis and effective therapy. The development of novel therapeutic strategies for ovarian cancer is therefore urgently needed. The HER-2/*neu* (also known as c-*erb*B-2 or NGL) gene is frequently amplified and/or overexpressed in a number of human cancers of glandular origin.[1–15] Overexpression of its protein product, p185, in breast,[1–3] ovarian,[4–7] and lung cancers[8–11] correlates with poor disease prognosis and relatively low patient survival rates. Cells with high-level p185 exhibit greater resistance to chemotherapeutic agents[16,17] and cytokines[18–20] and greater ability to metastasize.[21,22] Therefore, the HER-2/*neu* gene is an excellent target for development of therapeutic agents that could reverse malignant transformation of HER-2/*neu*–overexpressing cancer cells.

Our previous studies showed that simian virus 40 (SV40) large T antigen represses rat *neu* transcription in mouse fibroblast NIH3T3 cells.[23] A mutant SV40 large T antigen named K1, which, because of a single amino acid change within the retinoblastoma (Rb) binding/transformation domain cannot bind to Rb and fails to induce transformation,[24–26] represses rat *neu* as effectively as wild-type large T antigen and suppresses transformation of mutation-activated rat *neu* in the focus-forming assay. Although large T is able to induce transformation and is therefore inappropriate as a tumor suppression agent, the K1 mutant without transformation ability may be suitable for tumor suppression.[23,24] We therefore hypothesized that K1 may be able to suppress tumor development from HER-2/*neu*–overexpressing cancer cells through repression of HER-2/*neu* expression. Our results demonstrate that K1 is indeed a tumor suppressor gene in HER-2/*neu*–overexpressing ovarian cancer. By *in vivo* K1 gene transfer, the tumor-bearing mice were alive significantly longer, indicating that K1-liposome may function as a therapeutic agent for HER-2/*neu*–overexpressing ovarian cancer.

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MATERIALS AND METHODS

Cell lines and culture

SKOV-3, a human ovarian cancer cell line that overexpresses HER-2/*neu*,[7] was purchased from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium/F-12 medium (1:1, GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum.

DNA transient transfection

DNA transfections were carried out using the modified calcium phosphate precipitation procedure.[27] SKOV-3 cells were seeded

(10^6 in 10-cm dishes) the day before transfection. The cells were cotransfected with 5 μg of pNEUlit human HER-2/*neu* promoter linked to luciferase reporter construct[28] and 5, 10, or 15 μg of K1 plasmid DNA. Total DNA was equalized to 20 μg by control DNA pSV2E.[29] Cells were harvested 3 days later, and the luciferase activity of each transfectant was measured.[30]

Orthotopic human ovarian cancer nude mouse model

Four- to 6-week-old athymic female homozygous *nu/nu* mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind) and were cared for and used in accordance with institutional guidelines. Mice were housed for 1 to 2 weeks, and each mouse considered healthy was injected intraperitoneally (IP) with 2×10^6 SKOV-3 cells under aseptic conditions as described elsewhere.[31]

Liposome-mediated in vivo *gene transfer*

For measuring the *in vivo* transfection efficiency, a group of five female *nu/nu* mice were injected IP with 2×10^6 SKOV-3 cells. Six weeks later, a complex of 15 μg of pCHC–β-gal,[32] a plasmid encoding β-galactosidase, with 200 nmol of liposome[33] was injected IP into each mouse daily for 3 consecutive days. The mice were killed, and imprints of the tumors were made by touching the cross-section of the tissues onto the slices.

To test the therapeutic effects of K1-liposome complex on human ovarian cancer, the mice that had a tumor 5 days after IP injection of 2×10^6 SKOV-3 cells were placed into five groups. The mice in each group received weekly IP injections of 200 μL of a reagent containing 15 μg of K1 DNA complex with 200 nmol of liposome, 15 μg of control DNA (pSV2E) complex with 200 nmol of liposome, 15 μg of K1 DNA, 200 nmol of liposome, or phosphate-buffered saline (PBS). Treatment with the K1-liposome complex was discontinued after 7 months. The responses were observed for 1 year, and the survival durations of all the mice were recorded. Necropsies were performed, and tumor tissues and major organs (ie, brain, lung, liver, heart, kidney, and spleen) of the mice were collected for pathological hematoxylin-eosin staining, immunohistochemical staining, and biochemical analysis.

X-Gal staining

For X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining, the tumor imprints on the slides were fixed with a solution containing 0.05% glutaraldehyde and 2% formaldehyde for 5 minutes. They were washed three times with PBS and stained at 37°C for 2 hours with a solution of PBS containing 1 mg/mL X-Gal, 5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, and 2 mmol/L $MgCl_2$.

Immunohistochemical staining

Sections were taken from tumor samples and organs of tumor-bearing mice for histological analysis. After being fixed with formalin and embedded in paraffin, the sections were subjected to routine hematoxylin-eosin staining. p185 protein was detected by using polyclonal antibody (Dako, Carpinteria, Calif) as primary antibody, and biotinylated goat anti-rabbit immunoglobulin G (IgG) as second antibody, followed by incubation with streptavidin–alkaline phosphatase, and then developed in a phosphatase substrate kit; 1% methyl green was used as a counterstain. To detect K1 protein expression, monoclonal antibody against SV40 large T Ab-2 (Oncogene Science, Cambridge, Mass) biotinylated anti-mouse IgG was used, followed by incubation with ABC reagent (Vector, Burlingame, Calif), and developed in ABC chromogen substrate solution; Mayer's hematoxylin was used as a counterstain.

Immunoblotting

Immunoblot analyses on p185 protein in tumor cells were performed as described previously.[31] Protein samples (50 μg) from tumor tissues of dead mice were run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose filter, which was then incubated with primary antibody against p185 (c-*neu*–Ab-3, Oncogene Science), followed by horse peroxidase–conjugated goat anti-mouse IgG (Bio-Rad, Hercules, Calif), and protein was visualized with enhanced chemiluminescent detection reagents (Amersham, Arlington Heights, Ill) and exposed to x-ray films. The signals on the x-ray films were scanned into a computer with OneScanner-Ofoto. The ratio of p185 expression levels was quantitated by the quotient of band intensities determined using National Institutes of Health (NIH) Image V1.57.

Reverse transcription–polymerase chain reaction analysis of K1 expression

Total RNA was extracted from tumor tissues, large T stable transfectant BTn14[23] (as positive control for reverse transcription [RT]), or its parental cell, B104-1-1. Five micrograms of each RNA sample were reverse-transcribed to cDNA by standard procedures.[34] Amplification was performed on a Perkin-Elmer (Branchburg, NJ) DNA Cycler 480 in a total volume of 50 μL containing 5 μL of cDNA (or 20 ng of plasmid pK1 as positive control, or water as the negative control) in polymerase chain reaction (PCR) buffer (20 mmol/L Tris-HCl, pH 8.4; 25 mmol/L KCl; and 1.5 mmol/L $MgCl_2$), 50 mmol/L of each dNTP, 10 pmol of each primer, and 1 U of Taq polymerase (Promega, Madison, Mass). The primers are from within the large T coding region (5′–AAGATCTGCCATCTAGTGATGATGAG–3′ and 5′–ATC GATCTCTAGTCAAGGCAACTATAC–3′). Amplification was continued for 35 cycles by denaturing at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extending the primer at 72°C for 1 minute. The PCR products were analyzed by running on the 1.2% of agarose gel. Sequence analysis was performed to further prove the PCR products.

RESULTS

*K1 represses human HER-2/*neu *transcription in SKOV-3 cells*

We previously showed that K1 inhibits rat *neu* promoter activity and suppresses mutation-activated rat *neu* transformation.[23] To investigate whether K1 can also inhibit human HER-2/*neu* expression in human cancer cells, we examined the effects of K1 on human HER-2/*neu* promoter activity in human ovarian cancer cell line SKOV-3, in which HER-2/*neu* is overexpressed. Different amounts of K1 expression vector were transfected with human HER-2/*neu* promoter linked to luciferase reporter construct pNEUlit.[28,30] A dose-dependent decrease of luciferase activity through repression of HER-2/*neu* promoter was observed by the increasing concentrations of transfected K1, and a maximum of 85% inhibition was achieved (Fig 1). Thus, K1 can repress the activity of the human HER-2/*neu* promoter in SKOV-3 human ovarian cancer cells.

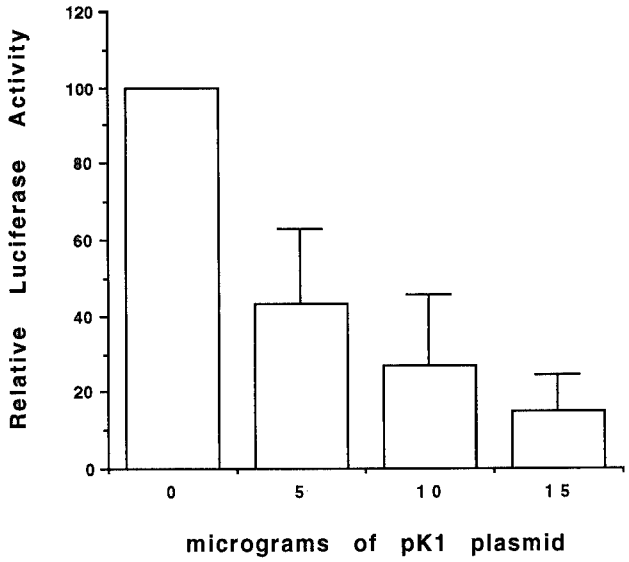

Figure 1. K1 represses human HER-2/*neu* promoter activity. Human SKOV-3 ovarian cancer cells were cotransfected with 5 μg of pNEUlit along with increasing amounts of K1 plasmid DNA. The luciferase activity of each transfectant was measured as described elsewhere.[29] Results from two experiments are represented. The bar indicates the standard deviation.

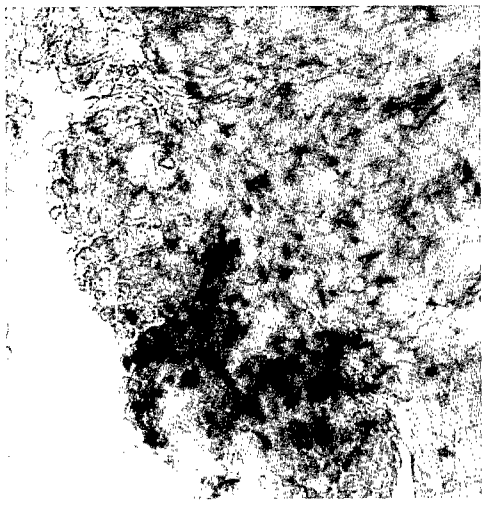

Figure 2. *In vivo* transfection efficiency of DNA-liposome complex. The tumor-bearing mice were injected with pCHC–β-gal–liposome complex (15 μg of DNA to 200 nmol of liposome) once a day for 3 consecutive days. Mice were killed 2 days later, and tumor sections were stained by X-Gal. Blue staining represents the cells that have been successfully transfected.

*K1 prolongs the survival of mice bearing HER-2/*neu*–overexpressing tumors*

To determine whether K1 suppresses *in vivo* tumor development in mice bearing HER-2/*neu*–overexpressing tumors, we used cationic liposome to deliver the plasmid encoding K1 into the tumor cells in an orthotopic ovarian cancer animal model.[31] To evaluate the *in vivo* gene transfection efficiency of the DNA-liposome complex in this animal model, pCHC–β-gal–liposome complex (in a ratio of 15 μg of DNA to 200 nmol of liposome) was injected IP daily for 3 consecutive days into a group of tumor-bearing mice, which had been injected IP with 2×10^6 SKOV-3 cells 6 weeks ago. An average of 27% of the tumor cells were stained positively with X-Gal. One representative stained region of a tumor sample is shown in Fig 2. As a negative control, another group of tumor-bearing mice were injected IP with 200 nmol of liposome only. No positively stained cells could be detected in these cells (data not shown). To detect the effect of K1-liposome complex on tumor development and mouse survival in this animal model, we induced tumor formation by injecting 2×10^6 SKOV-3 cells IP into 4- to 6-week-old female *nu/nu* mice; after 5 days, the tumor-bearing mice were separated into five groups. One group received weekly IP injections of the K1-liposome complex. The other four control groups of animals received an IP injection of PBS, control DNA pSV2E-liposome complex, K1 DNA, or liposome. The experiments were performed twice, and the cumulative results are shown in Fig 3.

The PBS-treated mice died within 3 months, while the mice of the other control groups died within 7 months. Treatment with K1-liposome complex was discontinued at the end of seventh month. The mice treated with K1-liposome complex survived significantly longer than the controls; about 40% were alive after 1 year. The autopsies showed that the mice from the control groups had large-volume (3- to 6-mL) malignant ascites and tumors within the peritoneal cavity or diaphragm or metastasis to the lungs. However, the mice that received K1-liposome complex had more locally distributed tumor nodules in their peritoneal cavities. This difference indicates that K1 suppressed the growth of HER-2/*neu*–overexpressing tumor cells so that tumors developed with longer latency. All of the mice survived for 1 year (ie, 40% of K1-liposome–treated mice) then were killed and examined for residual tumors, but no tumors were observed in the peritoneal cavity. Tissue sections from the major organs of the surviving mice (ie, brain, lung, heart, kidney, spleen, and liver) were analyzed by hematoxylin-eosin staining and found to be pathologically normal and free of microscopic tumor cells (data not shown). Our statistical analysis showed that the mice treated with K1-liposome complex survived significantly longer than those of the four control groups ($P < .05$), whereas none of the mice in the four control treatment groups survived significantly longer than any other ($P > .25$). Of all the treatments used in this study, K1-liposome complex is the only effective therapeutic strategy that prolonged the survival of mice bearing HER-2/*neu*–overexpressing tumors; 40% of the mice treated with K1-liposome complex were alive without tumors after 1 year.

K1 expression decreases the level of p185 in vivo

To determine whether the biological therapeutic effects of K1 *in vivo* were due to suppression of the HER-2/*neu*

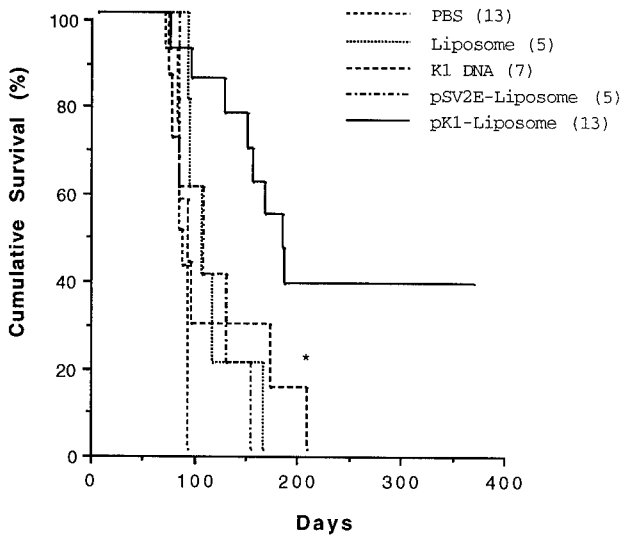

Figure 3. Survival of mice was prolonged after treatment with K1-liposome complex. Female *nu/nu* mice were injected intraperitoneally (IP) with 2×10^6 SKOV-3 cells 5 days before treatment. The mice received weekly IP injections of 200 μL of a reagent containing 15 μg of K1 DNA complex with 200 nmol of liposome, 15 μg of control DNA (pSV2E) complex with 200 nmol of liposome, 15 μg of K1 DNA, 200 nmol of liposome, or phosphate-buffered saline. The responses of the mice to treatment were observed for 1 year. The number of mice in each group is indicated. The asterisk marks the last injection.

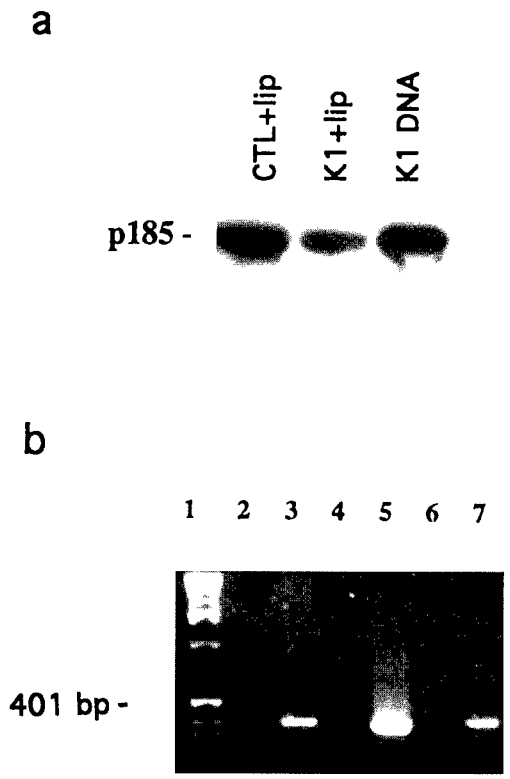

Figure 4. (a) HER-2/*neu*–encoded p185 protein expression level in tumor tissue. Protein samples from tumor tissues were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose. Blots were incubated with antibody against the p185 protein. Panel **a** shows one representative result. p185 expression levels in this specific experiment are pSV2E-liposome, 100%; K1-liposome complex, 57.8%; and K1 DNA, 98.0%. CTL+lip, control DNA pSV2E-liposome complex; K1+lip, K1-liposome complex; K1 DNA, K1 DNA only. The position of p185 is indicated. **(b)** Expression of K1 mRNA in tumor tissues. RNA was extracted from tumor tissues or large T stable transfectant BTn14[23] and its parental cell line, B104-1-1. Five micrograms of each RNA sample was reverse-transcribed, and polymerase chain reaction (PCR) was performed using a pair of primers from within the large T coding region; plasmid pK1 was used as positive control, while water as negative control. Lanes 1 through 7 represent a 1-kb DNA ladder, water, plasmid K1, B104-1-1, BTn14, pSV2E-liposome, and pK1-liposome, respectively. The 401-bp PCR product is indicated by the short dash.

p185 protein by the K1 gene product, histochemical and biochemical analyses were performed to examine the expression of K1 and p185 in the tumor tissues taken from the dead mice of the K1-liposome treatment group and the control groups. Hematoxylin-eosin staining confirmed that all of these tumors were adenocarcinomas (data not shown). Western blot analysis using antibody against p185 was performed to examine p185 expression in tumor samples from different groups. The average p185 expression level in the tumor samples of the K1-liposome–treated mice (not including the 40% tumor-free mice) was 54% (±8.6%) of that in the control groups. Figure 4a shows a representative set of these assays. RT-PCR experiments indicated that K1 mRNA was expressed in tumors of the K1-liposome complex treatment group (Fig 4b).

To detect p185 protein levels in individual tumor cells, immunohistochemical staining was performed on tumor tissue sections. In samples from K1-liposome–treated mice, K1 protein was expressed in approximately 60% of the cells and decreased p185 expression was easily detected in 50% of the total cells, while samples from other groups were negative for K1 and positive for p185 staining (Fig 5 and data not shown). Therefore, the decreased level of p185 in K1-liposome–treated tumor cells was most likely caused by K1 expression. Apparently, liposome-mediated K1 gene transfer exerted its therapeutic effects, most likely, through the repression of HER-2/*neu* p185. The reduction of p185 expression level by K1 suppressed the transforming activity of these tumor cells and delayed tumor development in mice. While 40% of the K1-liposome–treated mice were tumor free, the others developed tumors, which expressed 54% of p185, with longer latency and more localized distribution.

DISCUSSION

We previously showed that repression of HER-2/*neu* in HER-2/*neu*–overexpressing cancer cells results in loss of malignant phenotypes.[31,35–37] Since SV40 large T also represses HER-2/*neu* expression,[23] it is certainly inter-

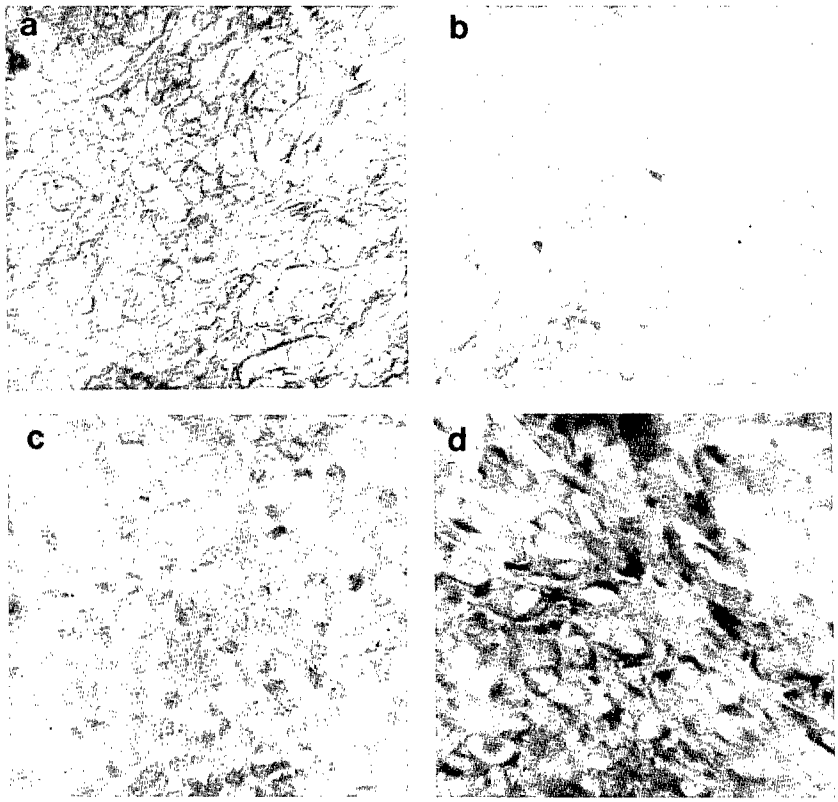

Figure 5. Immunohistochemical staining analysis of the p185 and pK1 protein expression in tumor cells. Polyclonal antibody for p185 and monoclonal antibody for SV40 large T were used for immunohistochemical staining on tumor sections. Panels **a** and **c** were representative tissue sections from the pSV2E-liposome treatment group. Panels **b** and **d** were representative tissue sections from the K1-liposome treatment group. Panels **a** and **b** were stained by p185 antibody; red represents positive staining. Panels **c** and **d** were stained by SV40 large T antibody; dark red represents positive staining.

esting to examine whether large T is able to suppress malignant phenotypes of HER-2/*neu*–overexpressing cancer cells. However, SV40 large T antigen is an oncoprotein with multiple functional domains. Three transformation domains—the Rb binding domain, the p53 binding domain, and the amino-terminal binding domain—are critical for its transformation of human cells.[38] Thus, it is not an appropriate therapeutic agent, even though it can repress HER-2/*neu* expression. The K1 mutant of SV40 large T, which contains a single point mutation in the Rb binding domain and is unable to bind to Rb protein, has been shown to lose its transformation activity.[23,24] Since K1 represses HER-2/*neu* expression as effectively as wild-type large T,[23] we tested whether K1 functions as a tumor suppressor for HER-2/*neu*–overexpressing ovarian cancer cells, and found that K1 represses HER-2/*neu* expression and has a significant therapeutic effect on mice with ovarian cancer. The 40% tumor-free survival rate shown in Fig 2 has a very important clinical implication, as patients with HER-2/*neu*–overexpressing ovarian cancers die within 2 years after diagnosis.[6] It will be interesting to further localize the region of large T critical for HER-2/*neu* repression. If a transforming region such as p53 binding domain is not required for HER-2/*neu* repression, a large T mutant lacking both Rb and p53 binding domains might have a better therapeutic effect than the K1 mutant. This important issue will be addressed in the near future.

We used a transient cotransfection assay to show that K1 represses HER-2/*neu* promoter activity in a dose-dependent manner; a maximum of 85% repression was observed (Fig 1). As we described previously,[39] because of the nature of the transient cotransfection assay, the percentage of repression based on this type of assay represents a low estimation. In the transfection assay, two plasmids such as pNEUlit and K1 are required. During transfection, some cells took both K1 and pNEUlit; the expression of luciferase in these cells was reduced by K1. However, some cells took only pNEUlit but not K1; these cells expressed high levels of luciferase, which resulted in high background for this type of assay. Thus, when we measured the luciferase activity from the whole cell population, the inhibitory effect of K1 on HER-2/*neu* promoter activity was diminished by cells expressing pNEUlit only, and the actual reduction of HER-2/*neu* promoter activity by K1 should have been more dramatic than what we detected in this assay. This point is further supported by the results shown in Fig 1. When the ratio of K1 to pNEUlit was increased, the percentage of cells with both K1 and pNEUlit transferred increased, and a more dramatic repression of promoter activity was detected.

Western blot and immunohistochemical analyses showed that the tumors from the K1-liposome–treated mice still expressed significant amounts of p185. This could be the result of limited transfection efficiency; that is, some tumor cells never received K1 DNA, and therefore p185 expression was unaffected. Alternatively, it could result from tumor heterogeneity; certain mutations that made cells resistant to K1-mediated p185 repression might have occurred in some tumor cells. These cells eventually developed into tumor masses and killed the mice. Despite of the mechanisms of this failure of K1-mediated p185 repression, the results shown in Fig 3 certainly represent a significant therapeutic efficacy. This approach has potential clinical implications for HER-2/*neu*–overexpressing cancers.

Toxicity is an important issue in liposome-mediated K1 gene therapy. Clinical chemistry, blood cell counts, organ histopathology, and pharmacokinetics should be systematically analyzed to address the adverse effects (if any) of this liposome system as a vehicle for the delivery of the gene. As a matter of fact, we have previously developed another HER-2/*neu*–targeting gene therapy strategy in which adenovirus 5 E1A is used as a tumor suppressor gene in the HER-2/*neu*–overexpressing human cancers.[31,37] To support the E1A phase I clinical trial protocol, systematic studies have been performed to detect the toxic effects of E1A-liposome injection. At the therapeutic effective dose of E1A-liposome (15 μg of DNA to 200 nmol of liposome), no adverse effects were observed on mouse kidney and liver functions, and no abnormal pathology was detected for major organs, including brain, heart, lung, liver, kidney, spleen, and ovary (X. Xing and M.-C. Hung, unpublished data). Since the gene delivery system, the dose of DNA-liposome, and the animal model of the E1A study are identical to those in the study reported here, it is unlikely that the liposome gene delivery system will produce significant toxicity, but whether K1 has any side effects must still be pursued.

There are many ways to inhibit oncogene expression in cancer cells, which, in turn, may inhibit malignant transformation of these cells. Antisense approaches represent a common and effective strategy to block expression of a specific oncogene in cancer cells. However, for cancers caused by oncogene overexpression, the antisense strategy may not be effective, because large amounts of antisense oligonucleotides or RNA must be generated in the target cells to effectively block the overexpressed mRNA of the specific oncogene. Repression of transcription may therefore be a more effective way of stopping malignant transformation than antisense approaches. Certainly, this might be true for HER-2/*neu*–overexpressing cancer cells, in which transcription upregulation plays a critical role in overexpression.[1,40] We previously showed that adenovirus 5 E1A can effectively repress HER-2/*neu* transcription and inhibit malignant transformation of HER-2/*neu*–overexpressing cancer cells.[35–37,41] In the study presented here, we further show that another transcriptional repressor, K1, a mutant of SV40 large T, also effectively represses HER-2/*neu* expression in tumors and prolongs survival of tumor-bearing mice. Our results provide experimental evidence that transcriptional repression is a good strategy for inhibiting oncogene expression and suppressing malignancy. This strategy may provide a unique and effective way for treating malignancies caused by oncogene overexpression.

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SHORT REPORT

Liposome-mediated *in vivo* E1A gene transfer suppressed dissemination of ovarian cancer cells that overexpress HER-2/*neu*

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The HER-2/*neu* proto-oncogene is frequently amplified or overexpressed in many different types of human cancers, a phenomenon that has been shown to correlate with shorter survival time and lower survival rate in ovarian cancer patients. We previously reported that increased HER-2/*neu* expression led to more severe malignancy and increased metastatic potential in animal models and that the adenovirus 5 *E1A* gene repressed HER-2/*neu* gene expression at transcriptional level and was able to suppress tumor growth when stably transfected into human ovarian cancer SKOV-3 cells which overexpress HER-2/*neu*. To investigate whether the *E1A* gene may be used as a therapeutic agent for HER-2/*neu*-overexpressing human cancers in living hosts, we first developed tumor-bearing mice by injecting SKOV-3 cells that overexpress HER-2/*neu* intraperitonealy into female nu/nu mice. Five days later, we used cationic liposomes to directly deliver the *E1A* gene into adenocarcinomas that developed in the peritoneal cavity and on the mesentery of the mice that received the SKOV-3 cell injection. We found that liposome-mediated *E1A* gene transfer significantly inhibited growth and dissemination of ovarian cancer cells that overexpress HER-2/*neu* in the treated mice; about 70% of these mice survived at least 365 days, whereas all the control mice that did not receive the gene therapy developed severe tumor symptoms and died within 160 days. The results suggest that liposome-mediated *E1A* gene transfer may serve as an effective therapy for human ovarian cancers that overexpress HER-2/*neu* by directly targeting the HER-2/*neu* oncogene.

Keywords: c-erbB-2; cationic liposome; tumor suppression; adenovirus-5 E1A; oncogene

The HER-2/*neu* gene (also known as c-*erb*B-2 or NGL) encodes a 185 kD epidermal growth factor receptor-related transmembrane protein (p185) with intrinsic tyrosine kinase activity (Padhy *et al.*, 1982; Schechter *et al.*, 1985; Bargmann *et al.*, 1986; Hung *et al.*, 1986; Ullrich and Schlessinger, 1990). The human HER-2/*neu* proto-oncogene is frequently amplified or overexpressed in many types of human cancers (Yu and Hung, 1995). Moreover, increased expression of HER-2/*neu* gene has been shown to correlate with the number of lymph node metastases in breast cancer patients (Slamon *et al.*, 1987) and shortened survival in breast, ovarian and lung cancer patients (Slamon *et al.*, 1989; Weiner *et al.*, 1990). Although there have been controversial clinical reports on the correlation between lymph node metastasis and the overexpression of HER-2/*neu* in breast cancer patients (Gusterson *et al.*, 1992; Toikkanen *et al.*, 1992), we recently provided experimental evidence that the mutation-activated rat *neu* oncogene can induce metastatic potential in mouse 3T3 cells and that enhanced expression of the human normal HER-2 gene can lead to increased metastatic potential in human non-small-cell lung carcinoma cells by promoting multiple steps in the metastatic cascade (Yu *et al.*, 1992, 1994; Yu and Hung, 1991). Our findings are consistent with the notion that the HER-2/*neu* gene plays important roles in the processes of tumorigenesis and cancer metastasis.

Ovarian carcinoma is the most lethal tumor of the female genital tract and continues to be the major cause of female cancer deaths, largely as a function of early abdominal seeding of this neoplasm, which produces carcinomatosis (Piver *et al.*, 1991). It has been estimated that more than 23 000 new cases of ovarian cancer will be diagnosed in the United States and 13 000 women will die of this disease each year (Boring *et al.*, 1992; Creasman and DiSaia, 1991). The 5 year survival rate of ovarian cancer patients increased only from 36% in 1975 to 39% in 1990; this lack of progress is due mainly to the difficulties in early detection of ovarian cancer, and the lack of highly effective treatment for advanced stages of the ovarian cancers when they can be detected. It is vitally important, therefore, to develop novel means of early detection and powerful treatments for later stages of these aggressive and devastating malignancies.

Previous studies demonstrated that amplification/overexpression of the HER-2/*neu* gene is a common phenomenon in primary ovarian cancers across different populations (Slamon *et al.*, 1989; Zhang *et al.* 1989; Berchuck *et al.*, 1990; Hung *et al.*, 1992). We have identified HER-2/*neu* gene overexpression in the SKOV-3 ovarian carcinoma cell line (Hung *et al.*, 1992) and found that the even higher level of HER-2/*neu* expression in the SKOV-3.ip1 ovarian cancer cell line, which was derived from SKOV-3 cells, correlates with more rapid progression of peritoneal carcinomatosis and a higher degree of malignancy than SKOV-3 cells. These observations suggest that HER-2/*neu* may serve as an excellent target of therapy for ovarian cancers that overexpress this gene.

In searching for repressors of the HER-2/*neu* gene, we discovered that when the adenovirus 5 *E1A* gene was stably transfected into cells, it can repress HER-2/

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neu oncogene expression and suppress the tumorigenic and metastatic potential of mutation-activated *neu* oncogene-transformed mouse 3T3 cells and dissemination of human ovarian cancer cells that overexpress HER-2/*neu* (Yu *et al*., 1990, 1991, 1992, 1993). In the study presented here, we investigated whether the *E1A* gene can act therapeutically as a tumor suppressor gene for cancers that overexpress HER-2/*neu* if introduced into living animals bearing such cancers that had disseminated, which mimic the later stage of human ovarian cancer (Beahrs *et al*., 1992). We used cationic liposomes to directly deliver the *E1A* gene into these tumors in mice by intraperitoneal (i.p.) injection of the liposome/E1A mixture and found that the treated mice survived significantly longer than the control mice that received no appropriate treatment. Our results indicate that liposome-mediated *E1A* gene transfer may become a new and effective gene therapy for human ovarian cancers that overexpress HER-2/*neu*, including later stage ovarian cancers.

Results

Establishment of ovarian cancer animal model

To investigate whether the *E1A* gene can be used to treat living animals bearing disseminated human ovarian cancers overexpressing HER-2/*neu*, we needed to first establish an animal model of such cancers, with which the effects of treatment can be examined. Because human ovarian cancer SKOV-3 cells (American Type Culture Collection) overexpress HER-2/*neu* mRNA at levels at least 100-fold higher than other cancer cell lines from the female genital tract (Hung *et al*., 1992), and can induce tumor formation and dissemination upon i.p. injection into nu/nu mice (Yu *et al*., 1993), we chose i.p. injection of SKOV-3 cells into nu/nu mice as an appropriate experimental approach for studying advanced human ovarian cancer. After injecting i.p. 2×10^6 SKOV-3 cells into individual female nu/nu mice, these mice were examined for tumor colonization and tumor dissemination by necropsy 1, 3 and 5 days postinjection. Several tumors > 0.5 mm in diameter were found on day 5 upon necropsy.

To examine whether these tumors were indeed induced by the injected SKOV-3 cells, we first stained them with hematoxylin-eosin and demonstrated that their pathologic identity was moderately differentiated adenocarcinomas (data not shown), which is consistent with the *in vitro* cytopathology of the SKOV-3 cell line and with the primary ovarian tumors from which the SKOV-3 cell lines was established. These tumors were further examined for HER-2/*neu*-encoded p185 expression by immunohistochemical staining using an avidin-biotinyl peroxidase technique (Hsu *et al*., 1982) with a primary polyclonal antibodies specifically recognizes p185 (1 : 300 dilution; Dakopatts Inc.). The protein was overexpressed in tumor sections obtained both from the mesentery (Figure 1A) and from the inner side of the abdominal wall (Figure 1B), which is also consistent with the p185 expression level of the SKOV-3 ovarian cancer cells. As negative controls, the same tumor sections reacted with anti E1A antibodies or anti large T antigen antibodies were stained negative (data not shown). These results demonstrated that nu/nu mice will produce disseminated human ovarian tumors that overexpress p185 five days after receiving i.p. injection of SKOV-3 cells,

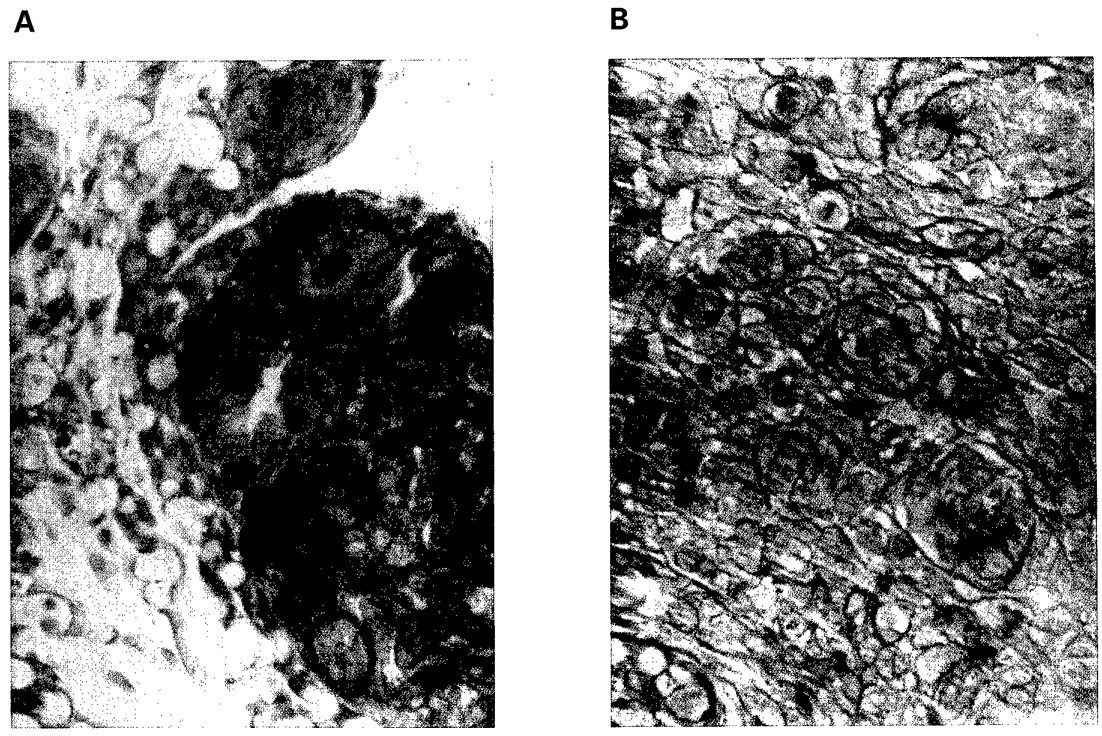

Figure 1 Immunohistochemical staining of p185 in tumors from (**A**) mesentery and (**B**) the inside of the peritoneal cavity. Representative slides with polyclonal anti-p185 antibodies (magnification × 40)

and that these tumor-bearing mice are suitable for further studies on the treatment effect of liposome-mediated *E1A* gene transfer.

Gene therapy for mice bearing ovarian tumors that overexpress p185

To find a gene delivery system that can effectively and directly transfer the *E1A* gene into tumors, we tested the efficiency of the cationic liposome, DC-Chol:DOPE {3β[N-(N′,N′-dimethylaminoethane)-carbamoyl] cholesterol:dioleoylphosphatidylethanolamine (3:2)}, in transfecting the lacZ gene into the SKOV-3 cells, since it has been shown to transfect mammalian cells efficiently with very low toxicity (Gao and Huang, 1991). We have found that DNA: liposome ratios of 1:10 and 1:15, which give high transfection efficienty and low toxicity, seem to be the optimal conditions for DC-Chol: DOPE liposome-mediated gene transfer *in vitro* (data not shown). Therefore, DNA:liposome ratio of 1: 13 (15 μg DNA: 200 nmole liposome) was further tested for its ability to transfer the lacZ gene dl *in vivo* into mice bearing tumors that overexpress p185. One week after three repeated i.p. injections of this DNA:liposome mixture, tumor-bearing mice were stained with X-gal and the tumors within the peritoneal cavity were stained blue (data not shown), indicating effective *in vivo* gene transfer. Therefore, the DNA:liposome ratio of 1:13 was used for *in vivo* liposome-mediated *E1A* gene transfer experiments.

Once the animal model and the optimal condition for DC-Chol: DOPE liposome-mediated gene transfer were established, we investigated the potential of using liposome-mediated *E1A* gene transfer as a therapy modality for ovarian tumors that overexpress p185. On day 5 after i.p. injection of SKOV-3 cells into nu/nu mice, these tumor-bearing mice were randomly divided into five groups. Group 1, the untreated control group, was intended to indicate the mortality of these mice without any treatment; group 2, the gene therapy treatment group, was initially given i.p. injections of the liposome/E1A DNA complexes (15 μg DNA and 200 nmol liposomes in serum free medium, total volume was 200 μl per injection); as other controls, group 3 was injected i.p. with *Efs* (pElAdl343 plasmid containing a 2 bp frame shift deletion in the *E1A* coding sequences) DNA:liposome complexes (15 μg DNA : 200 nmole liposome), group 4 was injected i.p. with 15 μg of *E1A* DNA only and group 5 was injected i.p. with 200 nmole liposome only. Each mouse from groups 2, 3, 4 and 5 received a repeated weekly i.p. injection. All mice were observed once a week for tumor development and then every other day when any or all of the following tumor symptoms appeared: abdominal bloating, loss of subcutaneous fat, hunched posture and decreased movement. Most of the mice died of tumor symptoms, some were killed when they appeared moribund or judging from our previous experience to be unlikely to survive more than 24 h.

Necropsies were performed on all of the mice when they died of tumor symptoms. Necropsies revealed typical tumor patterns in mice from all five groups, i.e., colonized tumor nodules on peritoneal and diaphragmatic surfaces; bowel, utero-ovarian, mesenteric, subsplenic, and subhepatic nodules; and large omental cakes of tumor and most of the mice developed bloody ascites (Table 1). Although all the dead mice developed similar tumor symptoms, invasions or tumor formations outside the peritoneal cavity were detected only in mice from the control groups and not in any mice trated with the *E1A*/liposome complexes (Table 1). One of the mice from the untreated group and one from the group given *E1A* DNA only died of tumor symptoms as early as 15 days after injection, and all the other mice from the control groups (groups 1, 3, 4 and 5) died within 160 days postinjection (Figure 2). Almost 70% of the mice treated with *E1A* DNA:liposome complexes, however, were still alive 365 days after injection. The difference in survival of mice treated with the *E1A* DNA:liposome complexes and of mice in the untreated group is highly significant ($P>0.01$, Figure 2). The *in vivo* E1A gene transfer experiments have been repeated two times with different batches of DNA preparation and similar results were obtained and were combined for analysis (Figure 2).

These results demonstrate that liposome-mediated *E1A* gene therapy can reduce the mortality of mice bearing human ovarian carcinomas that overexpress p185. In addition, the remaining eight mice from *E1A* DNA:liposome complexes treated group that survived

Table 1 Summary of necropsy findings

Symptoms	*1. Untreated*	*2.* E1a/*liposome*	*3.* Efs/*liposome*	*4.* E1A *DNA only*	*5. Liposome only*
Tumor dissemination sites	Abdominal wall, bowel, peritoneal cavity, ovary, liver, gall bladder, mesentery, diaphragm, spinal column	Abdominal wall, peritoneal cavity, mesentery, ovary, diaphragm	Abdominal wall, ovary, liver, mesentery, diaphragm, peritoneal cavity	Abdominal wall, bowel, ovary, uterus, liver, mesentery, diaphragm	Abdominal wall, peritoneal cavity, ovary, liver, mesentery, diaphragm
Tumour sizes (average), (cm^3)	1.75 ± 0.7	0.45 ± 0.32	0.7 ± 0.55	1.47 ± 0.9	0.3 ± 0.18
Tumor numbers (visible by eyes)	20 ± 9	5 ± 2	15 ± 8	19 ± 14	17 ± 6
Bloody Ascites, (ml)	3.7 ± 1.7	1.8 ± 0.6	1.9 ± 0.5	4.2 ± 2.9	3 ± 1.8
Invasion and Metastasis	Invasion into the spinal cord	Not detectable	Invasion into the spinal cord	Metastasized to the lungs, invasion into the motor nerves	Invasion into blood vessels
Other symptoms	Tumors surrounding gall bladder blocked bile secretion	Distended gall bladder	Severe weight loss	Severe weight loss, one or two legs paralyzed	Severe weight loss, rupture of blood vessels due to tumor invasion

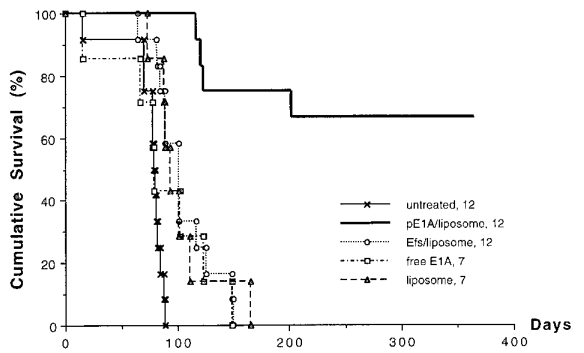

Figure 2 Mice treated with wild-type *E1A* : liposome complexes (heavy solid line) survived longer than untreated mice (solid line with crosses) or mice injected with wild-type *E1A* DNA alone, or with liposome alone, or with liposome plus *E1A* frame-shift DNA (*Efs*) (dotted lines) ($P<0.01$). Mice were given i.p. injections of 2×10^6 viable SKOV-3 human ovarian cancer cells 5 days before treatment. In two experiments, a total of seven or 12 mice made up each experimental group (as indicated in the figure notes). Mice were examined for tumor symptoms and were killed when they appeared moribund. Similar results were obtained from these experiments, and results were combined for analysis. The survival curves were obtained by recording the total survival days for each mice in different groups from the day of injection with SKOV-3 cells (day 1, 100% survival) to the days they died

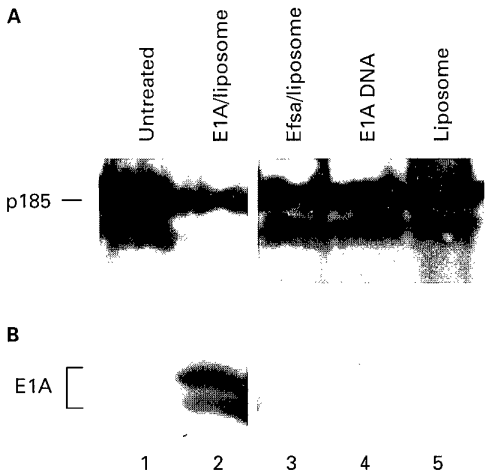

Figure 3 (**A**) Immunoblot analysis of HER-2/*neu*-encoded p185 proteins in tumor lysates from mice of each indicated group (groups 1 to 5, see Results section). Proteins from each sample (100 μg) were subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis prior to transfer to nitrocellulose filters. Filters were incubated with the primary antibody c-*neu*-Ab-3 against p185. The position of the p185 proteins is shown to the left. (**B**) E1A protein levels in tumor lysates from mice of each indicated group. Immunoblot analysis was done with m73 E1A-specific monoclonal antibody (Oncogene Sci. Inc.)

at least 365 days after therapy appeared normal and healthy and no tumor was found by necropsy on a randomly selected mouse out of the eight mice that survived. No microscopic lesion was found on pathologic examination of 5 μm serial sections of this mouse's organs, including mesentery, liver, stomach, kidney, ovary, spleen, intestine, pancreas, lungs and heart (data not shown). The results indicate that a significant fraction of treated mice can enjoy tumor-free survival after intensive liposome-mediated *E1A* gene therapy without noticeable side effects.

To investigate whether *E1A* DNA:liposome treatment will produce any significant side-effects, we examined the toxicites of *E1A* DNA:liposome on immunocompetent female ICR mice. The mice were injected with the effective treatment dose (15 μg *E1A* DNA: 200 nmol liposomes in serum free medium) for five consecutive days and their major organs were examined for pathological changes 6 weeks after injection. No abnormality was observed in liver, spleen, heart, brain, kidney and ovary of the five tested mice, except that two had slight focal penetration of lymphocytes in the lungs (data not shown). The results indicate that the effective dose of the *E1A* DNA:liposome that we used as gene therapy for ovarian tumors that overexpress p185 had no significantly toxicity and side-effect.

Expression of p185 in tumors obtained from mice necropsies

Since some mice treated with the *E1A* DNA:liposome complexes did form tumors (although with much longer latency and at lower frequency than the other control mice), we were interested in whether these tumors were similar biologically to the tumors from control mice. The tumors were excised from mice and examined histologically and biochemically. Histologic staining of frozen sections of these tumors revealed that the tumors from both the *E1A* DNA:liposome-treated group and the control groups were adenocarcinomas (data not shown). To measure the HER-2/*neu*-encoded p185 expression in these tumors, protein lysates were made from these and subjected to immunoblot analysis for the HER-2/*neu*-encoded p185 protein as previously described (Yu *et al*., 1993). The p185 proteins were readily detectable in all tumor samples from the control groups. However, p185 expression was repressed more than 50% in one small tumor (Figure 3A, lane 2) that was obtained from a mouse of the *E1A* DNA:liposome-treated group compared to the control groups. The heterogeneity of p185 protein is likely due to protein phosphorylation, which has recently been shown to produce heterogeneous protein bands in similar immunoblots (Kiyokawa *et al*., 1995). To investigate whether the tumors from mice treated with *E1A*:liposome complexes produced E1A proteins, we performed immunoblot analysis with anti-E1A antibodies (Figure 3B). The tumors from mice treated with E1A:liposome did express readily detectable levels of the E1A proteins (Figure 3, lanes 2), whereas none of the tumors from mice of those control groups expressed E1A proteins. Taken together, these results indicate that E1A expression were able to repress p185 expression in tumor cells from the *E1A* DNA:liposome-treated mice.

Discussion

One extremely important concern in gene therapy is the delivery system, for which liposomes have attracted much interest. Compared to retroviral vectors and adenovirus-associated vectors, the liposome delivery system has the advantages of easy handling, low toxicity and can be repeatedly used without eliciting

immune rejection response. As reported here, we have demonstrated that liposome-mediated *E1A* gene therapy for nude mice bearing ovarian cancers that overexpress HER-2/*neu* can lead to significantly prolonged survival and, in some cases, tumor-free survival. These results demonstrated the feasibility of using liposomes as *in vivo* delivery systems to transfer the *E1A* gene into these ovarian tumors in living hosts, a very significant progress toward the clinical application of *E1A* gene therapy for these ovarian cancers. Liposome-mediated gene transfer was recently reported for the treatment of melanomas in human (Nabel *et al.*, 1993). Taken together, these studies illustrate the efficacy of using liposomes as a delivery system for gene therapy of different types of human cancers.

In the study presented here, we obtained remarkable therapeutic effects using the condition of DNA:liposome ratio at 1:13. It is possible that even better therapeutic effects may be achieved with minor modifications. One possible explanation of the significant *in vivo* treatment effects may be that repeated *in vivo* injection may result in higher transfected:untransfected cell ratio, hence more tumor cells will receive *E1A* gene. Since another advantage of using liposomes for *in vivo* gene transfection is the possibility of targeting the encapsulated genes to specific cells where appropriate cytophilic ligands such as monoclonal antibodies are available, one of our next efforts should be designing liposomes that can target the *E1A* gene to tumors that overexpress p185 by incorporating into liposomes anti-p185 antibodies or the ligand for the HER-2/*neu*-encoded p185 receptor (when it becomes available).

The animal model used for this study has nude mice bearing disseminated human ovarian tumors that overexpress p185 five days after receiving i.p. injection of SKOV-3 cells. This animal model mimics stage III human ovarian cancers (Beahrs *et al.*, 1992), for which there is no effective therapeutic regimen. However, in the current study, liposome-mediated *E1A* gene therapy was able to effectively reduce the mortality of tumor-bearing mice and, in some cases, resulted in tumor-free survival, suggesting that liposome-mediated *E1A* gene therapy is a promising new therapeutic regimen for later stage ovarian cancers that overexpress HER-2/*neu*. In addition, this animal model will be very useful for future studies on searching effective therapies for later stage ovarian cancers.

The *E1A* gene of adenovirus-2, a close sera type of adenovirus-5, was shown to reduce the metastatic potential of *ras*-transformed rat embryo cells and to upregulate nm23 gene expression in the *ras*+*E1A* cells (Pozzatti *et al.*, 1988; Steeg *et al.*, 1988). We have also found that *E1A* can repress HER-2/*neu* gene expression and suppress the metastatic potential of HER-2/*neu*-transformed 3T3 cells by targeting the multisteps in the metastatic cascade (Yu *et al.*, 1992). In the study reported here, we observed metastasis in all the control groups but not in the *E1A*:liposome-treated group, suggesting that *E1A* has an *in vivo* metastasis suppressing function. Taken together, these studies strongly support the role of *E1A* as a metastasis suppressor gene.

We previously demonstrated that *E1A* products can dramatically inhibit HER-2/*neu*-mRNA level and HER-2/*neu* p185 expression in human breast cancer cell lines (Yu *et al.*, 1990; Yan *et al.*, 1991); we have also shown that the *E1A* gene products can repress HER-2/*neu* gene expression at the transcriptional level by targeting a specific DNA element in the HER-2/*neu* gene promoter (Yu *et al.*, 1990). It is likely, therefore, that the *E1A* tumor suppressing function is due to transcriptional repression of the overexpressed HER-2/*neu* gene, which may be one of the diverse molecular mechanisms that account for the tumor suppressor function of *E1A* in SKOV-3 ovarian cancer cells. However, other molecular mechanisms could also contribute to *E1A* tumor suppressing function *in vivo*. For example, it has been shown that adenovirus *E1A* can render hamster cell lines more susceptible to lysis by natural killer cells and macrophages (Cook and Lewis, 1984; Sawada *et al.*, 1985). It has also been reported that *E1A* can induce an increased sensitivity to cytotoxicity by tumor necrosis factor in transfected NIH3T3 cells (Cook *et al.*, 1989). E1A protein was recently shown to induce cytotoxic responses that resembles programmed cell death (apoptosis) (Rao *et al.*, 1992) and E1A has been reported to convert three unrelated types of human cancer cells into a nontransformed state (Frisch, 1991), which suggests that *E1A* may also function as a tumor suppressor gene for certain human cancer cells in which HER-2/*neu* is not overexpressed. Despite the potential involvement of different molecular mechanisms, our results have at least demonstrated that *E1A* may be a useful therapeutic agent for the treatment of human cancers that overexpress HER-2/*neu*.

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